THE PROTEIN REQUIREMENTS OF COLLEGE WOMEN ON HIGH CEREAL DIETS WITH OBSERVATIONS ON THE ADEQUACY OF SHORT BALANCE PERIODS [1, 2]

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THREE FIGURES

(Received for publication September 3, 1948)

Nitrogen balance studies have been used routinely in assessing the protein requirements of adults. The criterion of adequacy has been the establishment of nitrogen equilibrium. In conducting these studies, short periods have generally been used. Sherman ('20) reported the protein requirement of one subject consuming bread protein for 5 consecutive three-day tests, as well as data for several other subjects on diets for an indeterminate number of days. Murlin et al. ('46a, '46b) in a series of studies on nitrogen metabolism, routinely employed three- to 5-day periods in comparing the value of various nitrogen constituents for maintaining nitrogen equilibrium in man. Hegsted et al. ('46) studied the protein requirements of adults using periods of 6 days or longer. Bricker, Mitchell and Kinsman ('45) in testing protein requirements used 5 to 12 days, depending on uniformity of balances. The present

[1] A preliminary report of these data was presented before the American Institute of Nutrition in Atlantic City, March, 1948.

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investigation was undertaken to estimate the validity of short time studies in predicting the protein requirement of adults for a longer period, i.e., 10 weeks.

EXPERIMENTAL PROCEDURE

General plan

The individual protein requirements of 9 college women consuming a diet in which cereals supplied 70% of the protein were estimated in the initial phases of this study by short balance periods. The efficacy of the diet for maintaining the subjects in good health was tested in a subsequent 10-week period, during which 10 subjects (the original nine, plus an additional subject) were fed according to the protein requirements assessed for them. Constancy of nitrogen balance data, body weight, hematological data and performance tests were used to assess adequacy of dietary protein.

Subjects

Ten college women (9 for the entire study and one additional during the last 70 days), 19 to 30 years of age, selected on the basis of health and willingness to cooperate, served as subjects. Health status was determined by examinations by the University Health Service medical officers. The initial age, height, weight and surface area of the individual subjects are shown in table 3.

Dietary regime

The diets were composed of a basal diet low in nitrogen plus the protein mixtures being tested. The low nitrogen foods and the quantities consumed by one of the subjects are listed in table 1. Sugar and butterfat, in proportions to supply one-third of the calories as fat, were used to adjust individual caloric needs. Maintenance of body weight was used as the criterion of caloric adequacy. In general, 45 cal. of food per kilogram of body weight per day sufficed for weight maintenance at all levels of nitrogen intake. Subjects D, F, and H

TABLE 1

Sample daily basal diet of a 48 kg subject together with calculated[1] *values for fat and caloric content*

COMPONENT	AMOUNT	ENERGY VALUE	FAT
	gm	*Cal.*	*gm*
Cornstarch cookies:			
Cornstarch	196.5	692	...
Sugar	19.5	78	...
Butterfat[2]	37.5	338	37.5
Corn oil	3.8	34	3.8
Agar, powdered	3.0	...	...
Salt mixture[3]	16.4	...	...
Lemon juice	100.0	33	...
Sugar	158.0	632	...
Butterfat[2]	39.0	351	39.0
Total		2158	80.3
Applesauce[4]	100.0	80	...
Lettuce[4]	30.0	5	...
French dressing[4]	15.0	90	10.0
Total		175	10.0
Vitamin supplement[5]			

[1] Bradley ('42).

[2] Washed free of salt and curd.

[3] For formula see footnote 4, bottom of page.

[4] Omitted in period 3.

[5] Supplied by two Stuart Formula Tablets daily (Stuart Laboratories, Pasadena, California). These furnished the following: thiamine chloride, 4.0 mg; riboflavin, 4.0 mg; nicotinamide, 30.0 mg; calcium pantothenate, 5.0 mg; pyridoxine, 0.2 mg; ascorbic acid, 100.0 mg; mixed tocopherols, 4.0 mg; vitamin A, 5000 USP units; vitamin D, 800 USP units.

were exceptions, the first two requiring only 35 cal. and the latter 50 cal. per kilogram.

The salt mixture[4] incorporated into the cookies was devised to supply the mineral needs of the subjects and to

[4] This salt mixture was made according to a formula supplied by Dr. W. C. Rose, Department of Biochemistry. It contained 5.6 gm baking powder, 3.75 gm mineral salts, and 7.1 gm NaCl. The baking powder, made in this laboratory, contained 27.0% $NaHCO_3$, 37.6% $Ca(H_2PO_4)_2.H_2O$, and 35.4% cornstarch. The mineral salts in the proportions used were: $CaCO_3$, 32.14; $KHCO_3$, 50.18; $MgSO_4 \cdot 7H_2O$, 13.78; $FeC_6H_5O_7 \cdot 3H_2O$, 3.72; $MnSO_4 \cdot 4H_2O$, 0.0472; $CuSO_4 \cdot 5H_2O$, 0.1670; and KI, 0.0094.

serve as a leavening agent for the cookies. The high levels of riboflavin and nicotinamide were given as a precaution against the possibility that lowered protein intakes might increase riboflavin requirements (Oldham and co-workers, '47) or that lowered tryptophan intakes incident to low protein intakes might increase niacin requirements (Krehl et al., '45).

The protein mixture chosen for study (see table 2) was one devised in connection with another study on the relative protein value of diets in which 30 or 70% of the protein was of cereal origin. The 70% cereal protein diet, as the latter diet will be termed hereafter, was selected for this long term study

TABLE 2

Quantities fed and protein contribution of protein foods used in estimation of individual nitrogen requirements

SOURCE OF PROTEIN	WEIGHT OF FOOD		PROTEIN CONTRIBUTED
	Period 1	Period 2	
	gm	*gm*	%
Cereal foods			
White bread, water	61.5	123.0	59.5
Oats, rolled	7.0	14.0	10.5
Non-cereal foods			
Beef, lean ground	7.4	14.8	14.7
Cream, 20%	43.4	86.8	13.4
Potatoes, white	9.0	18.0	1.9

because its use would allow the inclusion of a larger volume of natural foods than would a protein mixture composed predominantly of proteins of animal origin. This inclusion of natural foods was deemed desirable to avoid monotony in an otherwise uninteresting, purified diet. The reasoning behind the choice of types and proportions of proteins is set forth in a paper dealing with a comparison of the 70% and 30% cereal proteins (Bricker and Smith, unpublished). The composition of the protein mixture and the per cent protein contributed by the individual foods are shown in table 2. The percentage of total protein supplied by each of the foods was as follows: white bread, 59.5; oatmeal, 10.5; beef, 14.7;

cream, 13.4; and potato, 1.9. When these foods were added to the basal diet, adjustments were made so that fat continued to supply one-third of the total calories (which were also adjusted to supply 45 cal./kg body weight).

TABLE 3

Age, body size and basal metabolism of 10 women subjects on a study of protein requirements

SUBJECT	AGE	BODY WEIGHT	STANDING HEIGHT	SURFACE AREA [1]	BASAL METABOLISM [2]
	years	*kg*	*cm*	m^2	*cal./24 hr.*
A	24	46	164.5	1.48	1257
B	19	67	170.8	1.78	1486
C	24	55	157.5	1.55	1197
D	30	58	167.0	1.65	1245
F	25	76	180.3	1.96	1469
G	22	48	156.8	1.45	1010
H	22	50	162.6	1.52	1200
I	24	66	167.0	1.74	1249
J	26	70	165.7	1.78	1248
K	22	65	173.4	1.78	1343

[1] Estimated by the DuBois ('16) formula, $\text{Wt.}_{kg}^{0.425} \times \text{Ht.}_{cm}^{0.725} \times 71.84$.

[2] Determined by oxygen consumption measured by the McKesson Waterless Metabolor.

The lengths of the three initial feeding periods described below were those shown by previous experience to allow sufficient adjustment to the new dietary regime.

Order of feeding

Four test periods, 10 to 70 days in length, were used in the order shown below:

Period 1 — 70% cereal protein diet supplying 1.6 gm nitrogen, 10 days.

Period 2 — 70% cereal protein diet supplying 3.2 gm nitrogen, 10 days.

Period 3 — Low protein diet supplying 0.2 gm nitrogen, 15 days.

Period 4 — 70% cereal protein diet fed according to individual nitrogen requirements, 70 days.

During the first 10-day period, 4 subjects (A, F, G, and J) were placed on the 1.6 gm intake and 5 subjects (B, C, D, H, and I) on the higher level of 3.2 gm of nitrogen. They were fed in reverse order for the next 10 days. The period ended on December 19, prior to the Christmas holiday when subjects were allowed free choice of food. Period 3, extending from January 4 to 19, was followed by a two-day break in which the subjects consumed an ordinary mixed diet supplying 50 gm protein daily. Period 4 extended from February 13 to April 23. This period was postponed in order to allow time for analysis of samples and data necessary for computation of individual protein needs. Two test periods intervened between periods 3 and 4. During this intervening time the subjects received 3.0 gm nitrogen daily from bread or meat, the order of feeding depending on the individual schedule. Subject K started on the study at the beginning of period 3.

Calculation of individual protein requirements

The individual requirements used in constructing the diets fed during the 10-week period were calculated from the data obtained during the first three periods. These requirements were estimated according to the technique described in the first paragraph of the section entitled Results.

Collection and preparation of samples

Foods were divided for analysis into 4 sample groups, as follows: cookies; lemon juice, lettuce, and applesauce; bread and oatmeal; and potatoes, meat, and cream. The samples were pooled in 5-day composites except during period 4, when 7-day pools were made.

Twenty-four hour urine samples were collected and preserved with 1 to 2% acetic acid. Fecal samples were marked with ferric oxide or chromic oxide. Fecal collections were made during the last 5 days of periods 1 and 2, during the last 10 days of period 3, and in weekly intervals throughout period 4.

As soon as foods were sampled and feces collected they were refrigerated. After pooling, the samples were acidified with 50 to 100 ml glacial acetic acid, blended in a Waring Blendor and made up to a given weight with distilled water.

Methods of assay

All nitrogen analyses were made by the Kjeldahl method. Hematologic determinations were made weekly on fasting capillary blood. Hemoglobin was determined colorimetrically by the acid hematin method of Newcomer. Erythrocytes were counted by an accepted technique (Gradwohl, '43). For these counts the blood was diluted with Hayem's solution and an improved Neubauer counting chamber calibrated by the National Bureau of Standards was used.

Performance tests

In this portion of the study tests were used (with the exception of the Rohrschach) which have been reported by one or more authors as being sensitive to dietary deficiency or change. The critical fusion frequency of flicker, speed of tapping, coordination time and manual steadiness in holding a static position were studied according to the techniques listed by Keeton et al. ('46). Physical endurance was measured by use of a recording electrodynamic brake bicycle ergometer (Kelso and Hellebrandt, '34) according to the following procedure: the subject rode against resistance of 6 amperes at the rate required to maintain an output of 4 volts until unable to pedal further. After a 10-minute rest the test was repeated. Graphic recordings of the work output were made by an electric kymograph. The endurance score was expressed as the total number of seconds during which the desired voltage was maintained.

Other tests used in an attempt to determine possible effects of the diet on mental function and personality were proofreading, memory, code learning, reading, and Rohrschach multiple choice tests. The techniques and forms used are described below:

Proofreading. This test was fashioned after Thurstones' "Scattered X'S" (Thurstone and Thurstone, '41), which they classed as a test of the preceptual factor of intelligence. Tests were performed the first, third, 5th, 7th, and 9th weeks of the study.

Memory tests. These tests, given during the weeks 1, 2, 4, 6, 8, and 9, were also made according to a form reported by Thurstone ('37), containing lists of numbers and objects, numbers alone, and names with initials to be memorized. Two modified forms were constructed in which names and numbers of objects were changed.

Code learning. The test form used[5] here consisted of 4 parts, covering number checking, code translation, finger dexterity, and counting. The same form was used throughout and tests were made during the first, third, 5th, 7th, and last weeks of the experiment. Harrell ('43) has reported that thiamine-fed subjects improved significantly more in performance of this test than did controls fed placebos.

Reading tests. The tests chosen here were the advanced Iowa Silent Reading Tests, forms Am, Bm, Cm, and Dm.[6] The parts measuring rate and comprehension were used in tests during the first, 4th, 7th, and 10th weeks. One form was used in each of the 4 test in the order listed above.

Rohrschach. This test, given during the first, 6th, and 10th weeks, was made by using "Harrower-Erickson Multiple Choice Test" blanks (Harrower-Erickson and Steiner, '45) with the Rohrschach cards.

It was deemed wise to gauge the extent of a learning factor by conducting the above performance tests for the same length of time on a control group of subjects on freely chosen diets. Nine subjects ranging in age from 20 to 26 years, seniors in nutrition, were selected on the basis of interest in the study and willingness to cooperate. These subjects were not fed weighed diets but selected their diets according to the

[5] Test 2, Code Translation from the "Survey of Working Speed and Accuracy" constructed and standardized by Floyd Ruch, California Test Bureau, 1943.

[6] Iowa Silent Reading Tests, new edition published 1943 by the World Book Company, Chicago, Illinois.

pattern recommended by the Food and Nutrition Board of the National Research Council.[7] Good cooperation in achieving this pattern was evidenced by special efforts made to secure foods not provided by their regular diets. These girls will be referred to in the discussion as control subjects and those on weighed diets as protein subjects.

RESULTS OF THE INVESTIGATIONS

The nitrogen balance data of periods 1, 2, and 3 plotted against the nitrogen intakes for 9 of the subjects are shown in figure 1. Subject K did not participate in this part of the study. Data at the 3.2 gm intake are missing on the graphs for subjects F and J since they were ill with influenza at the time. Regression lines have been fitted to these points by the method of least squares. The resulting regression equations shown on the individual graphs have been used to assess the amount of food nitrogen needed for equilibrium as well as the amount required for "adult growth" (Bricker et al., '45). The net amount of nitrogen needed for "adult growth" was estimated by allowing 0.77 gm nitrogen per square meter of surface area. The gross amount of nitrogen was obtained by substituting these net values as Y in the individual regression equations shown for each subject on figure 1. The values thus obtained represent the total amount of dietary nitrogen required for equilibrium and "adult growth." These values for each subject are presented in table 4. Translated in terms of protein (N $\times$ 6.25), the amounts required (all subjects except J) in grams were 31.7, 35.2, 27.1, 30.2, 30.9, 26.9, 29.4, 42.2, and 32.0, with an average of 31.7 gm per day. The value for J was not included in the average since it was shown later to be an underestimate.

The average daily nitrogen balances and body weights of the subjects for each week of the 10-week period are shown in figure 2 and the average for all subjects (except J) in figure 3. The balances remained positive throughout the

[7] Circular no. 122, Food and Nutrition Board, National Research Council, August, 1945, p. 16, list I.

10 weeks for all subjects except J and D, with no tendency for a less positive balance at the end of the period. It will be seen that the ingestion of the quantity of protein estimated to be needed by subject J resulted in values around equili-

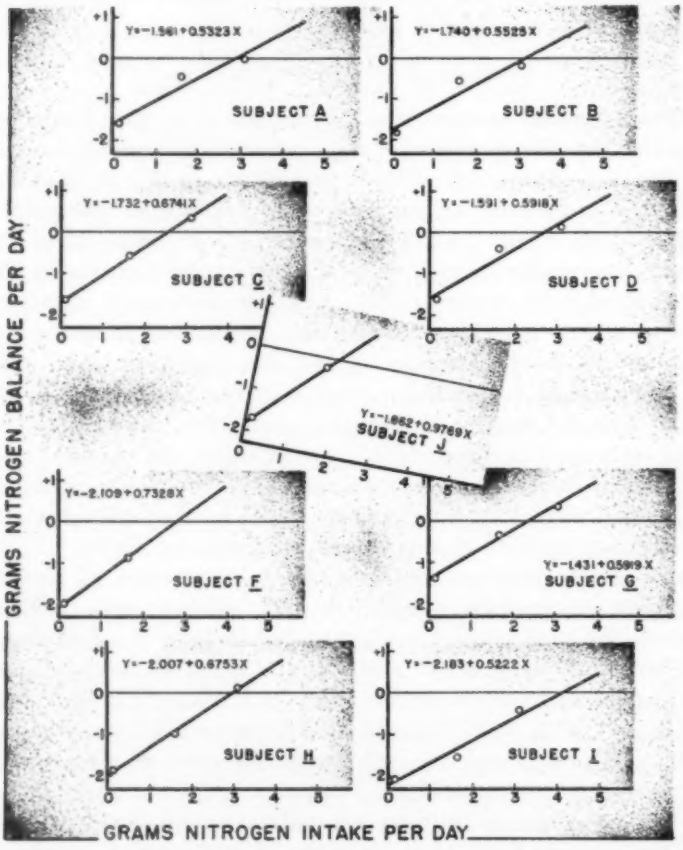

Fig. 1 Individual nitrogen balance data used in estimating the requirements of 9 college women on mixed diets are plotted on the above graphs. Regression lines fitted to the data by the method of least squares and the equations for these lines appear on each. The diet contained mixed proteins in amounts to furnish 70% of the protein from cereal.

brium with no consistently positive balance as was the case with the other subjects. This error in estimation is not surprising, since the estimate was based on two instead of three nitrogen intakes. When the intake for this subject was increased at the 8th week positive balances consistently resulted. The negative balance recorded for subject D during the 8th week can be accounted for in part by poor fecal separation as evidenced by a low value (0.436 gm nitrogen) in week 7 followed by a high value (0.944 gm nitrogen) in week 8.

TABLE 4

Estimated individual nitrogen needs on a 70% cereal protein diet

SUBJECT	PROTEIN INDEX [1]	NITROGEN FOR "ADULT GROWTH"	NITROGEN REQUIREMENT [2]	DAILY PROTEIN REQUIREMENT		
				Per person [3]	Per m^2	Per 1000 basal cal.
	%	*gm/day*	*gm/day*	*gm*	*gm*	*gm*
A	53.2	1.14	5.08	31.7	21.4	25.2
B	55.2	1.37	5.63	35.2	19.8	23.7
C	67.4	1.19	4.33	27.1	17.5	22.6
D	59.2	1.27	4.83	30.2	18.3	24.3
F	73.3	1.51	4.94	30.9	15.8	21.0
G	59.2	1.12	4.31	26.9	18.6	26.6
H	67.5	1.17	4.70	29.4	19.3	24.5
I	52.2	1.34	6.75	42.2	24.3	33.8
J	(97.7)	(1.37)	(3.31)	(20.7)	(11.6)	(16.6)
K [4]	65.0	1.37	5.13	32.0	18.0	23.8
Average (except J) [5]	61.4	1.28	5.08	31.7	19.2	25.1

[1] Nutritive value as indicated by the *b* values of the regression equations (fig. 1). This is equivalent to the coefficient of true digestibility × biological value.

[2] Calculated by solving for Y in the $Y = a + bX$ equation when $X =$ gm nitrogen needed for "adult growth."

[3] Nitrogen requirement × 6.25.

[4] Subject K did not participate during periods 1 and 2. The average nutritive index of the other 9 subjects was used as the *b* value and her total nitrogen excretion on the low nitrogen diet as the *a* value, thus giving an approximated equation of $Y = -1.961 + 0.6500\ X$, from which the daily nitrogen requirement of 5.126 gm was estimated.

[5] Estimated nitrogen requirements of J appear too low on the basis of continued negative nitrogen balances when the estimated quantities were fed.

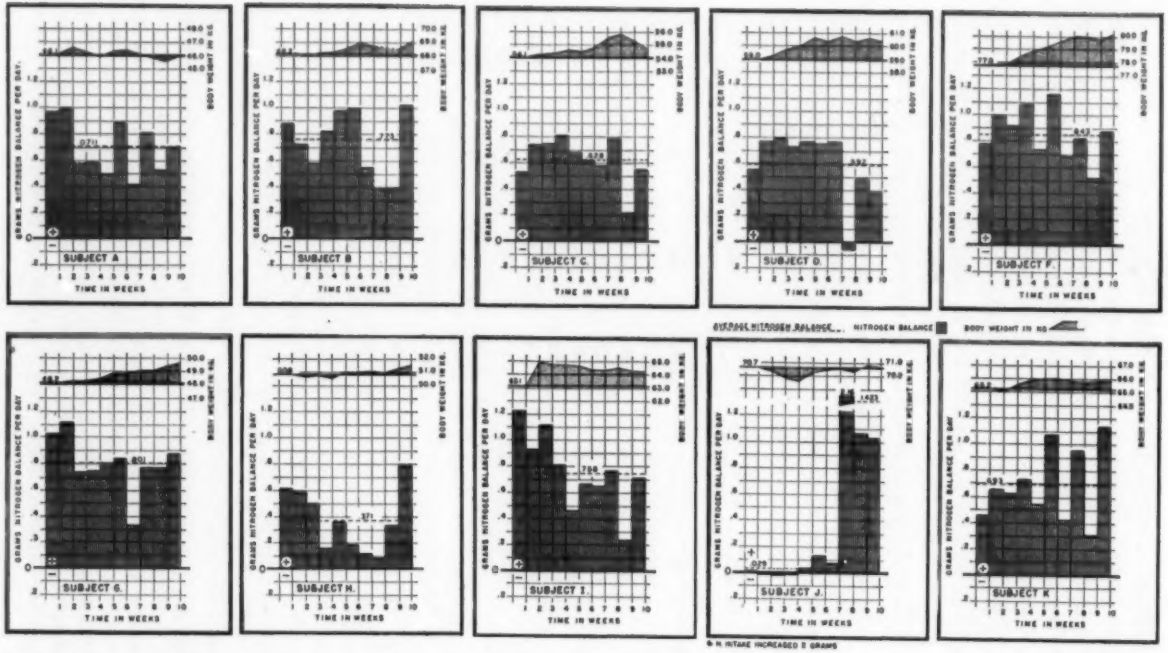

Fig. 2 Average body weights and average daily nitrogen balance (7-day balance periods) for 10 subjects fed estimated protein requirements for 10 weeks (note data are missing during weeks 3 and 8 for subjects A and B respectively).

Weekly hemoglobin values and erythrocyte counts for period 4, presented in table 5, showed some intra-subject variation but no upward or downward trend throughout the whole period. The average 10-week values for the 10 subjects ranged from 12.3 to 14.0 gm per 100 ml for hemoglobin and 4.39 and 4.99 million per mm³ for erythrocyte counts. These hemoglobin and erythrocyte values are within the normal range cited by Ohlson et al. ('44). These authors reported

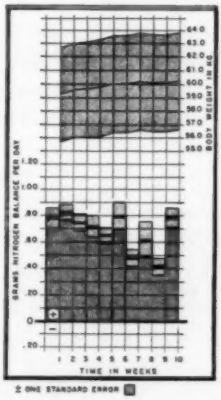

Fig. 3 Average of data shown in figure 2 for all subjects except J, together with the standard error of each mean.

hemoglobin values between 12.2 and 14.6 for 72% and erythrocyte counts of 4.18 to 4.95 for 71% of their cases. In the present study the white cell counts and packed cell volumes remained constant and within normal levels through the 10 weeks.

The performance tests at the beginning showed no consistent difference between the protein and control subjects, nor was there any indication of change in function according to these criteria after 10 weeks on the 70% cereal diet.

DISCUSSION OF RESULTS

The constancy of the apparent nitrogen stores as evidenced by the continued positive balances over the 10-week period

suggests that the estimates made by the short time experimental periods supplied ample protein, except for one subject (J). Further study of the data reveals that the average apparent nitrogen retention (average positive balance) is 0.37

TABLE 5

Weekly hemoglobin values and erythrocyte counts of 10 subjects for period 4

SUBJECT	WEEKS										Average
	1	2	3	4	5	6	7	8	9	10	
	Hemoglobin (gm/100 ml)										
A	13.1	13.0	14.1	14.0	13.4	13.6	14.0	14.8	14.2	14.0	13.8
B	13.6	14.1	13.7	13.4	13.7	14.0	14.1	...	12.2	13.9	13.6
C	14.0	12.9	12.6	12.8	13.2	12.7	12.5	12.2	13.7	13.4	13.0
D	13.0	14.0	12.8	12.8	12.8	12.9	13.3	14.0	13.5	13.7	13.3
F	13.6	13.9	13.3	14.1	14.2	14.0	14.3	14.0	13.6	14.0	13.9
G	11.8	13.1	12.0	12.1	12.3	12.1	12.2	12.4	12.7	12.0	12.3
H	13.7	14.1	13.2	13.7	13.9	13.9	13.9	15.1	14.6	14.3	14.0
I	13.6	13.2	13.9	13.6	13.9	13.2	13.8	14.4	13.7	13.6	13.7
J	12.1	12.5	13.4	13.2	13.3	12.8	13.2	13.7	13.1	14.1	13.1
K	13.7	14.1	13.7	13.6	13.2	13.0	13.5	13.4	13.0	13.9	13.5
Average	13.2	13.5	13.3	13.3	13.4	13.2	13.5	13.8	13.4	13.7	
	Erythrocyte count (millions per mm^3)										
A	4.86	4.79	4.92	4.90	4.49	4.71	4.72	5.02	5.00	4.97	4.83
B	4.73	4.85	4.93	5.12	4.71	4.87	4.84	...	4.71	4.98	4.86
C	4.66	4.19	4.36	4.49	4.86	4.55	3.80	4.00	4.44	4.56	4.39
D	4.62	4.76	4.77	4.83	4.77	4.74	4.84	4.98	4.83	4.80	4.79
F	4.68	4.71	4.90	4.84	4.83	4.85	4.85	4.75	4.86	4.89	4.82
G	4.63	4.69	4.65	4.47	4.69	4.77	4.69	4.76	4.42	4.50	4.63
H	4.94	4.73	4.89	5.23	4.92	5.10	4.86	5.25	4.89	5.13	4.99
I	4.66	4.68	4.61	4.80	4.81	4.81	4.79	4.89	4.68	4.72	4.74
J	4.82	4.84	4.79	4.98	4.96	4.86	4.71	5.10	4.65	4.73	4.84
K	4.93	4.86	4.57	4.72	4.65	4.75	4.56	4.51	4.32	4.53	4.64
Average	4.75	4.71	4.74	4.84	4.77	4.80	4.67	4.81	4.68	4.78	

gm per square meter of surface area, whereas the figure used in constructing the individual protein need was 0.77 gm. This latter figure was derived from studies on men fed about 90 gm of protein daily continuously for 220 days (Grindley, '12). The indicated lower adult growth need of the girls may

have been apparent but not real, due either to an accumulated deficit of nitrogen during preceding periods or to the fact that the estimated protein need was too low to allow maximum nitrogen retentions. It is also possible that a combination of these factors may have operated. The continued positive deviation from equilibrium indicates to the authors that there is an "adult growth" need for protein nitrogen the exact amount of which has not been conclusively demonstrated in this experiment.

It is interesting to speculate as to the extent to which accumulated nitrogen deficits may have accounted for the continued positive balance. The deficits were approximated by calculating the algebraic sum of the observed and estimated [8] nitrogen balances which accumulated from the end of the Christmas holiday to the beginning of the 10-week period. The accumulated positive balances used in making this estimation were obtained by totalling the balances during the 10-week period. Accumulated positive nitrogen balances during the 10-week period were considerably greater than the nitrogen deficits in 7 of the 8 subjects for whom data were available, the average positive balance for these 7 being 51 and the negative deficit 30 gm. Subject H, the exception, had an estimated deficit of 33 and a positive storage of only 26 gm.

These data are not sufficiently conclusive to postulate a sex difference in adult growth needs, since the possibility that a higher intake of the same protein might have induced greater positive balances has not been tested. One should not ignore, on the other hand, the possibility that some of the subjects might have shown the same degree of positive balance on lower protein intakes.

The average estimated daily protein need of 31.7 gm (all subjects but J) amounts to 3.1 gm nitrogen per square meter. This is slightly above the recommendation of Hegsted et al.

[8] Estimations were essential for the first days of the period, since fecal analyses were not made. Average fecal values assayed for the last portion of the period were applied to the beginning. For the two days in which the subjects received 50 gm of protein an average storage of 4.0 gm was allowed.

('46), who estimated a nitrogen need of 2.9 gm per square meter when the nitrogen was supplied by an all-vegetable diet in which 62% of the protein was of cereal origin. A somewhat lower value, 2.4 gm, was suggested when the same diet was used and one-third the protein of each food was replaced by meat. Sherman ('20) suggests a minimum requirement of 0.5 gm protein per kilogram of body weight. When the data herein reported are expressed on a body weight basis a similar value, i.e., 0.55 gm protein per kilogram, is obtained. Neither Hegsted et al. nor Sherman considered the needs for adult growth in their estimates. If the present study had omitted such needs, the average daily protein requirement would have been 8 gm less (6.25 $\times$ 1.28, average daily nitrogen requirement for adult growth). Using regression lines of nitrogen balances on nitrogen intakes for subjects on typical self-chosen dietaries, Leverton and Marsh ('42), in a study of 70 college women, proposed a minimum daily protein requirement of 50 gm per person, and Leitch and Duckworth ('37), from a compilation of published data, suggested 48.2 to 52.4 gm as the average daily requirement. Both of these suggestions are considerably above the daily requirement of 31.7 gm indicated by the present study.

In the case of subject K, a fairly satisfactory estimate was made of the nitrogen need by using average utilization and surface area data. This was evidenced by continuous retention of nitrogen in amounts similar to those retained by other subjects.

The protein index of the protein mixture (biological value times coefficient of true digestibility) averages 61.4 $\pm$ 2.4[9] for all subjects except J. This compares well with the value of 58.6 $\pm$ 5.9 for 5 subjects on a mixed diet reported by Bricker et al. ('45).

The constancy of the hemoglobin and erythrocyte counts throughout the 10-week period shows that the diet was adequate for maintenance of cell color and number. Leverton

[9] In this instance, and in all other cases, the value defining the significance of the mean is the standard error.

et al. ('44) have suggested that for hemoglobin regeneration intakes of at least 75 gm of protein daily are desirable, with the additional recommendation that generous amounts of animal protein be included. Obviously the process of hemoglogin maintenance should not require as generous amounts of protein as does hemoglobin regeneration.

Statistical evaluation of the data on performance tests showed no deterioration of function during the 10-week dietary period for either the protein or the control groups. In those tests where consistent improvement was noted, i.e., in memory, reading, proofreading, and clerical tests, the degree of change was not significantly different [10] between the protein and control subjects.

The initial performances of the two groups of subjects on the ergometer test were not significantly different when compared by the method of Fisher for unpaired data (Fisher, '46). Average initial scores with their standard errors were 124.8 ± 19.0 and 176.8 ± 16.4 seconds for protein and control groups, respectively. Similar comparisons for the final scores of 140.0 ± 17.2 and 159.9 ± 16.0 seconds also revealed no real difference. Application of the method of Student ('25) for the analysis of paired data to initial and final endurance scores of the protein and the control groups revealed no significant trends in muscular endurance during the 10-week period of observation.

The data for 4 of the tests — critical fusion frequency of flicker, speed of tapping, coordination time, and manual steadiness in holding a static position — were evaluated by expressing them as coefficients of comparison according to the method of Keeton et al. ('46). These coefficients are the differences between initial and final scores expressed as a multiple (or fraction) of the standard deviation for all subjects on the initial test. In none of the 4 tests mentioned above was the coefficient of comparison significantly different for the two groups of subjects and consequently the coefficients of scoring,

[10] Probability of a chance outcome was greater than 0.05.

which represented the average coefficient of comparison for each group, showed an insignificant difference between the protein and control subjects.

For the remaining performance tests (proofreading, memory, code learning, reading, and Rohrschach), the change from the initial to the final score was expressed as a percentage of the initial score. These per cent scores were then combined and compared by the method of Fisher ('46) for unpaired groups of data. The results of this comparison showed insignificant differences in all instances. Thus, though both groups had uniformly improved in all of the tests except the Rohrschach, the rate of improvement appeared to be unaffected by the dietary treatments imposed.

SUMMARY AND CONCLUSIONS

Ten college girls, 19 to 30 years of age, cooperated in a study of the protein needs of adults. During an experimental period of 105 days, divided into 4 periods, the following were determined: (1) nitrogen balances on a low protein diet; (2) individual protein needs for nitrogen equilibrium and estimated "adult growth" needs when subjects received a diet in which 70% of the protein was of cereal origin; and (3) adequacy over a 10-week period of these estimated protein needs for maintenance of nitrogen equilibrium and physical well-being (as indicated by three psychomotor tests, 5 tests of preceptual and intellective function, and one test of physical endurance).

The results were as follows:

1. The average protein index (biological value $\times$ true digestibility) of the protein mixture composed of beef, bread, 20% cream, oatmeal and potatoes was 61.4 $\pm$ 2.4 for 9 subjects for whom satisfactory data were available.

2. The quantities of protein from the above protein mixture needed for nitrogen equilibrium plus the amounts calculated to be needed for "adult growth" were found to be 31.7, 35.2, 27.1, 30.2, 30.9, 26.9, 29.4, 42.2, and 20.7 gm per day for each of 9 subjects, with an average of 30.5. A re-

quirement of 32.0 gm was estimated for a 10th subject on the basis of average utilization figures.

3. Nine of the 10 subjects were in positive nitrogen balance throughout the 10-week period during which the estimated amounts of protein were fed. The other subject, for whom the smallest indicated requirement was secured in the previous periods, showed some negative balances until the intake was increased.

4. Hematologic data and results of performance tests failed to reveal any deterioration of physiological functioning during the 10 weeks on estimated protein requirement levels.

It may be concluded from these studies: (1) that for diets of the type used, containing predominant proportions of cereal proteins, the average daily requirement for adult maintenance and growth is 31.7 $\pm$ 1.6 gm per person or 25 gm per 1000 cal. of basal heat; and (2) that the protein requirements computed from experimental periods of such length that the urinary nitrogen attains a relatively constant level are valid for periods of at least 70 days.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Mr. Nathaniel Glickman, University of Illinois Medical School, for arranging for the loan of psychomotor equipment and for training the technician who conducted the tests; to Mrs. Metta Zahorsky for conduct of the performance tests; and to Dr. Lela Booher, nutritionist, General Mills, Inc., for her personal interest and advice.

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THE NEW VITAMIN A REFERENCE STANDARD AND ITS USE IN EVALUATING THE VITAMIN A POTENCY OF FISH OILS[1, 2]

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ONE FIGURE

(Received for publication September 13, 1948)

Ever since vitamin A became recognized as an essential in the normal nutrition of man and of domesticated animals, attempts have been made to develop methods which may be used to measure this nutrient quantitatively. The methods of vitamin A analysis developed thus far have been of three general types; namely, biological, physical, and chemico-physical. Since these methods are either expensive, time-consuming, or lacking in specificity, there has been a continuous attempt to improve them and to develop new methods of assay. That the problem has not been satisfactorily solved is illustrated by recent reviews of existing methods of vitamin A analysis (Association of Vitamin Chemists, Inc., '47; Dann, '47; and Nelson and DeWitt, '47). In all probability, some of the difficulty encountered in the past has been due to the vitamin A standards then in use.

[1] Authorized for publication on August 11, 1948, as Paper No. 1462 in the Journal Series of The Pennsylvania Agricultural Experiment Station. This investigation was supported in part by a grant from The Nutrition Foundation, Inc.

[2] Some of the data were taken from the thesis of the senior author submitted to The Graduate School of The Pennsylvania State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Gridgeman ('44) has presented many objections to the use of the U.S.P. Reference Cod Liver Oils (no. 1 and no. 2) and of the International Standard β-carotene as vitamin A standards. More recently evidence has appeared in the literature concerning variability of the potency of different bottles of the U.S.P. Reference Cod Liver Oil no. 2 (U. S. D. A., '47). However, in 1943, U.S.P. Reference Cod Liver Oil no. 2 was replaced by U.S.P. Reference Cod Liver Oil no. 3, which in turn has recently been replaced by a new U.S.P. Vitamin A Reference Standard (crystalline vitamin A acetate dissolved in cottonseed oil, U.S.P. Vitamin Advisory Board, '47b). Furthermore, the factor used in converting physical data to biological potency has been a source of confusion. The two generally accepted conversion factors are 2000 (in the United States) and 1600 (in England).

In view of these facts, it seemed highly desirable that the relationship between the International Standard (β-carotene), U.S.P. Referencce Cod Liver Oil no. 3, the new U.S.P. Reference Standard, and pure crystalline vitamin A acetate be established through simultaneous biological assays. To clarify the situation further with respect to conversion factors, it also appeared desirable that vitamin A assays be conducted simultaneously on a number of representative commercial fish oils. It was with these objectives in mind that the experiments herewith reported were initiated.

EXPERIMENTAL PROCEDURES

In order to correlate spectrophotometric with biological data, the extinction coefficients were determined for each of the vitamin A standards and for each of the fish oils by means of the Beckman quartz spectrophotometer, using isopropanol as the solvent.

The vegetable oil used to dilute the various vitamin A supplements preliminary to feeding was a refined cottonseed oil having a peroxide value of 2.4 (milli-equivalents of peroxide oxygen per 1000 gm of oil) as determined by the method of Greenbank and Holm ('34). The vitamin A supplements were

made up in 100 ml portions and stored in several small, glass-stoppered, amber-colored bottles, each of which was flushed with CO_2 after the addition of the solution. In this connection it should be stated that the β-carotene solutions were prepared by first dissolving the weighed amount of International Standard β-carotene in 2 ml of C. P. benzene and then adding the oil. Since the benzene was not evaporated from the oil, its final concentration in the assay solutions was 0.38%, 0.58% and 0.77% in the solutions containing 10, 15, and 20 I.U. per milliliter, respectively. The dilute solutions of vitamin A and β-carotene used in the biological assays were stored at 5°C. during the feeding period. These solutions were brought to room temperature at the time of feeding and then immediately returned to the refrigerator. It was found that vitamin A and β-carotene were stable over a period of at least two weeks when the oil solutions were handled in the above manner. The stability of the vitamin A in the assay solutions was checked at frequent intervals by adding 9 ml of the Carr-Price ('26) reagent to 1 ml of the oil solution and determining the resulting $L_{1\,cm}^{1\%}$ —620 mμ value. The stability of carotene in similar solutions was checked by adding 10 ml of petroleum ether to 1 ml of the oil solution and determining the $L_{1\,cm}^{1\%}$ —440 mμ value. These determinations were made by means of the Evelyn photoelectric colorimeter.

The biological assay procedure used was essentially that set forth by the U. S. Pharmacopoeia Vitamin Advisory Board ('47a), except that the various standards were fed at multiple levels. Rats from our own stock colony were from 21 to 28 days of age and weighed from 40 to 50 gm when placed on the vitamin A-deficient diet. The rats purchased from a biological supply house were of unknown age but ranged from 40 to 50 gm in weight when placed on the vitamin-deficient diet. All test animals were maintained in individual, all-metal cages during both the depletion and curative periods.

The basal vitamin A-free diet consisted of 18% casein (hot alcohol extracted), 65% corn starch (dextrinized), 8% yeast,[4]

[4] Anheuser-Busch Strain G, irradiated.

5% hydrogenated vegetable oil,[5] and 4% salt mixture.[6] When the test animals had been depleted of their body reserves of vitamin A, they were allocated to the various assay groups in such a manner as to secure the best possible distribution by litter, sex, body weight, and state of depletion. The feeding of the vitamin A supplements was started on the day the animals were assigned to the respective groups and was continued for a 4-week period. The oily solution of the supplements was fed in small porcelain fraction cups at the rate of 0.1 ml per day. In order to insure immediate consumption of the vitamin A fractions, one or two drops of a sterilized solution of rice polish extract (20% solution of desiccated rice polish concentrate) was added to the oil supplements in each fraction cup. The biological assays were carried out in two comparable series, i.e., about half of the total number of animals were used during the first period and after the assays on these had been completed other comparable groups of animals were used. The condensed results of these studies are presented in the tables and figures which appear in this paper.

RESULTS AND DISCUSSION

Extinction coefficients of the vitamin A standards

The extinction coefficients of each of the standards used in the biological assays are presented in table 1. A comparison of these values with those reported in the literature is of interest. Oser et al. ('43) reported $E^{1\%}_{1\,cm}$ —325 mμ values for the whole and unsaponifiable fractions of U.S.P. Reference Cod Liver Oil no. 3 in isopropanol to be 1.14 and 0.98, respectively. Since our values were considerably lower than these and since similar instruments and solvents were used in the two investigations, it appears that the oil lost some of its vitamin A potency during the interim period. When one takes into consideration the matter of solvent differences the values obtained in these studies for the U.S.P. vitamin A

[5] Crisco.
[6] McCollum's no. 185.

acetate in oil (5.24 and 5.25) compare favorably with the mean values (5.37 and 5.28) for the whole and unsaponifiable fractions, respectively, obtained in connection with the recent U.S.P. collaborative study (U.S.P. Vitamin Advisory Board, '47a). The value of 1520 obtained for the crystalline vitamin A acetate prepared in this laboratory is somewhat higher than the value (1510) reported by Baxter and Robeson ('42) for this ester of the vitamin when dissolved in ethyl alcohol, but is the same as that reported by Guerrant et al. ('48).

TABLE 1

Extinction coefficients of the vitamin A standards used in the biological assays

STANDARD	SOLVENT	WAVE LENGTH	$E^{1\%}_{1\ cm}$
U.S.P. Reference Cod Liver Oil no. 3	Isopropanol	325 mμ	0.914
	Isopropanol	325 mμ	0.817 [1]
New U.S.P. Vitamin A Reference Standard	Isopropanol	325 mμ	5.24
	Isopropanol	325 mμ	5.25 [1]
Crystalline vitamin A acetate prepared in this laboratory	Isopropanol	325 mμ	1520
International Standard (β-carotene)	Petroleum ether	450 mμ	2300

[1] Nonsaponifiable fraction.

Extinction coefficients reported in the literature for pure β-carotene (Gridgeman, '44; Devine et al., '45; Guerrant et al., '48) are generally somewhat higher than the coefficient found for the International Standard used in this investigation and this suggests that the latter is no longer the purest form of β-carotene obtainable (Van Genderen and Van Eekelen, '44; Wilkinson, '41).

Results of the biological assays

Some of the pertinent biological data obtained during the course of assaying the vitamin A standards and the 27 representative fish oils used are presented in tables 2, 3, and 4.

TABLE 2

Comparative biological responses of the various groups of rats used in assaying the vitamin A standards and the 27 representative fish oils

ASSAY GROUP	SOURCE OF VITAMIN A	ESTIMATED DOSAGE [1]	MALES	FEMALES	AVERAGE INITIAL WEIGHT	DEPLETION PERIOD		AVERAGE GAIN IN WEIGHT DURING ASSAY				
						Average duration	Average weight at end	Males	Females	Males and females		Standard deviation [2]
										Unweighted [2]	Weighted [2]	
		units/day	no.	no.	*gm*	*days*	*gm*	*gm*	*gm*	*gm*	*gm*	
1	New U.S.P. Vitamin A Reference Standard	0.75	7	8	45	27.9	103	16.6	22.8	19.9	19.7	11.2
2	New U.S.P. Vitamin A Reference Standard	1.0	7	6	46	27.8	106	19.6	23.2	21.2	21.4	8.5
3	New U.S.P. Vitamin A Reference Standard	1.5	7	8	46	27.6	101	31.6	30.6	31.1	31.1	10.3
4	New U.S.P. Vitamin A Reference Standard	2.0	8	8	46	27.7	104	36.0	35.8	35.9	35.9	7.3
5	U.S.P. Reference Cod liver oil no. 3	1.0	5	8	44	27.4	100	10.0	26.3	20.0	18.1	13.6
6	U.S.P. Reference Cod liver oil no. 3	1.5	7	7	46	27.8	102	23.3	30.6	26.9	26.9	9.3
7	U.S.P. Reference Cod liver oil no. 3	2.0	8	8	46	29.6	104	29.3	27.5	28.4	28.4	9.6
8	International Standard	1.0	3	7	44	26.7	94	25.3	23.3	23.9	24.3	7.9
9	International Standard	1.5	8	8	45	27.2	104	36.5	24.5	30.5	30.5	11.0
10	International Standard	2.0	8	7	46	27.7	108	41.6	32.6	37.4	37.1	9.0
11	Vitamin A acetate prepared in this laboratory	1.0	7	6	46	28.4	106	21.1	25.5	23.2	23.3	7.7
12		1.5	7	8	44	27.9	101	32.1	30.9	31.5	31.5	6.6
13	Negative control	. .	6	6	45	27.6	102	. .	. .	. .	. .	. .
14	Swordfish liver oil, domestic [4]	1.5	4	7	44	29.4	108	32.5	22.5	28.5	27.5	7.2
15	Swordfish liver oil, imported [4]	1.5	5	7	46	29.1	104	37.2	28.1	31.9	32.7	12.5
16	Skip Jack liver oil [4]	1.5	4	7	46	29.0	104	31.3	21.9	25.3	26.6	10.7
17	Skip Jack liver oil, imported [4]	1.5	4	7	46	28.5	108	25.8	29.6	28.2	27.7	7.9
18	Bluefin tuna liver oil [4]	1.5	3	6	45	29.4	106	27.3	21.8	23.7	24.6	6.7
19	Bluefin tuna liver oil, imported [4]	2.0	3	7	45	28.5	110	31.0	26.6	27.9	28.8	5.6
20	Totuava liver oil [4]	1.5	5	6	45	29.6	106	19.4	24.2	22.0	21.8	10.3
21	Totuava liver oil, imported [4]	1.5	4	7	44	29.8	102	32.3	18.1	23.3	25.2	10.4
22	Albacore liver oil, no. 1 [4]	1.5	5	7	48	29.6	112	41.2	25.8	31.9	33.2	13.4
23	Albacore liver oil, no. 2 [4]	1.5	5	6	45	30.3	108	25.8	28.5	27.3	27.2	13.7
24	Barracuda liver oil [4]	1.5	4	6	48	29.7	109	11.8	26.2	20.4	19.0	10.9
25	Black cod liver oil [4]	1.5	4	7	46	29.4	105	31.8	27.7	29.2	29.7	9.5
26	Bonita liver oil [4]	1.5	4	7	45	28.5	107	31.0	28.6	29.5	29.8	7.8
27	Halibut liver oil [4]	2.0	4	5	47	29.5	112	18.5	30.4	25.1	24.5	14.7
28	Jewfish liver oil [4]	2.0	5	6	46	29.4	111	36.6	32.8	34.5	34.7	9.2
29	Ling cod liver oil [4]	1.5	4	7	45	29.5	105	16.0	23.0	20.5	19.5	9.0
30	Mackerel liver oil [4]	1.5	4	7	45	28.0	106	34.8	24.0	27.9	29.4	10.2
31	Menuke liver oil, imported [4]	1.5	5	7	45	28.2	106	28.6	24.3	26.1	26.4	8.2
32	Sebastus Marinus liver oil [4]	1.5	5	6	46	28.0	105	26.0	40.3	33.8	33.2	12.4
33	Spearfish liver oil, imported [4]	1.5	4	6	45	28.5	107	21.0	30.7	26.9	25.9	12.2
34	Yellow Tail liver oil [4]	1.5	4	7	45	28.1	105	29.5	25.1	26.7	27.3	8.8
35	Argentine shark oil [5]	2.0	4	6	44	35.7	127	30.7	29.3	29.9	30.0	10.2
36	Dogfish liver oil [5]	2.0	4	6	45	34.9	123	45.0	30.7	36.4	37.8	10.1
37	Blue shark oil [5]	2.0	5	5	45	35.0	127	19.2	20.8	20.0	20.0	11.0
38	Fortified cod liver oil [5]	2.0	4	6	44	36.4	127	29.2	24.0	26.1	26.6	6.4
39	Soupfin shark oil [5]	2.0	4	7	45	35.8	128	25.5	30.4	28.6	28.0	5.6
40	Mexican shark oil [5]	2.0	5	6	45	34.9	125	28.8	30.2	29.5	29.5	10.9

[1] Based on spectrophotometric data.

[2] For sex.

[3] Based upon the weighted average gain in weight during assay.

[4] Furnished through the courtesy of Mead Johnson and Company, Evansville, Indiana.

[5] Furnished through the courtesy of Distillation Products, Inc., Rochester, New York.

It is to be noted that some of the oils were fed at the 1.5 unit level while others were fed at the 2.0 unit level (see table 2). This was because preliminary assays had shown the estimated potency of certain oils, based on spectrophotometric data, to be too high; hence an increased amount of some of the other oils was fed during the biological assay.

There was no evidence by the colorimetric method of assay of a loss of either vitamin A or carotene from the diluted oil solutions during a 120-day storage period under the conditions

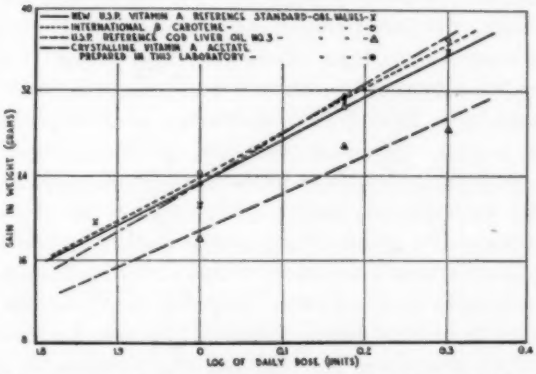
NEW U.S.P. VITAMIN A REFERENCE STANDARD—OBS. VALUES—X
INTERNATIONAL β CAROTENE—
U.S.P. REFERENCE COD LIVER OIL NO.3—
CRYSTALLINE VITAMIN A ACETATE, PREPARED IN THIS LABORATORY—
GAIN IN WEIGHT (GRAMS)
LOG OF DAILY DOSE (UNITS)

Fig. 1 Response graphs for the different vitamin A standards. The points represent the observed responses, while the lines represent the calculated responses. Here it is to be noted that U.S.P. Reference Cod Liver Oil no. 3 was definitely less effective in promoting growth, per unit of vitamin, than was the vitamin A acetate or the International Standard (β-carotene).

of these experiments. It appears, therefore, that the usual practice of preparing fresh solutions of the vitamin A standards and the vitamin A carriers at weekly intervals is not always necessary, provided a satisfactory solvent oil is used and provided the vitamin solutions are properly handled and stored.

On the basis of data presented in table 2 (groups 1 to 12, inclusive) it is possible to compare the biological responses resulting from feeding various definite amounts of the different vitamin A standards. In setting up the biological tests it

[1] For sex.
[2] Based upon the weighted average gain in weight during assay.
[3] Furnished through the courtesy of Mead Johnson and Company, Evansville, Indiana.
[4] Furnished through the courtesy of Distillation Products, Inc., Rochester, New York.

was originally planned that all test groups would be composed of equal numbers of comparable animals, but owing to the fact that some animals died before the assays were completed, the data presented in table 2 do not show this experimental design. However, in order to compensate partially for unequal sex distribution the average growth response of each assay group was weighted for sex in making the final computations. The dose-response equations were derived according to the method of Coward ('38) and by means of these equations growth responses were calculated. The calculated responses as well as the observed responses are illustrated in figure 1. The equations for the lines given are: New U.S.P. Vitamin A Reference Standard: $y = 40.5x + 23.4$; International Standard: $y = 42.1x + 23.9$; U.S.P. Reference Cod Liver Oil no. 3: $y = 46.9x + 23.3$. In each formula y equals the growth response in grams during the 28-day assay period and x equals the log (to the base 10) of the daily dose of the vitamin.

The potency of a given vitamin A standard in terms of the other standards was evaluated by substituting the calculated growth response (the growth response represented by the appropriate equation, using the 1.0, 1.5, and 2.0 unit levels, respectively) and then determining the arithmetic mean of the three values thus obtained. Thus, in terms of the International Standard (β-carotene), the new U.S.P. Vitamin A Reference Standard was found to contain 9610 I.U. per gram, the U.S.P. Reference Cod Liver Oil no. 3 to contain 1220 I.U. per gram, and the crystalline vitamin A acetate to contain 2.93×10^6 I.U. per gram. In terms of the new U.S.P. Vitamin A Reference Standard, the crystalline vitamin A acetate was found to contain 3.045×10^6 U.S.P. units per gram, while the U.S.P. Reference Cod Liver Oil no. 3 contained 1250 U.S.P. units per gram. In other words, the U.S.P. unit in the new U.S.P. Vitamin A Reference Standard was found to be 1.36 times as active biologically as was the U.S.P. unit in the former standard (U.S.P. Reference Cod Liver Oil no. 3) when this standard was assumed to contain 1700 U.S.P. units per gram. The potency observed for the U.S.P. Reference Cod Liver Oil

no. 3 (1220 I.U. per gram) is less than that reported as being the average potency (1365 I.U. per gram) obtained in the U.S.P. Vitamin A Collaborative Study of 1946–1947 (U.S.P. Vitamin Advisory Board, '47a). However, both values definitely indicate that the U.S.P. unit as found in the U.S.P. Reference Cod Liver Oil no. 3 and the International unit do not represent the same amount of biological activity. With the introduction of the new U.S.P. Vitamin A Standard, the International unit and the U.S.P. unit may be considered to be equal in biological potency. The fact that U.S.P. Reference Cod Liver Oils no. 2 and no. 3 deteriorated in biological potency during the course of their usage accounts for many of the discrepancies in the literature concerning the potency of certain vitamin A carriers, and perhaps for some of the variation in reported conversion factors.

The potency of the crystalline vitamin A acetate prepared in this laboratory compares favorably with that in the new U.S.P. Vitamin A Reference Standard. Calculated as the alcohol, the potency found for this crystalline vitamin A acetate was 3.492×10^6 U.S.P. units per gram. This value is in relatively good agreement with those of 3.181×10^6 and 3.424×10^6 I.U. per gram reported by Mead, Underhill and Coward ('39) for vitamin A alcohol when examined in the form of the carboxylate and naphthoate esters, respectively. If one multiplies 3.492×10^6 U.S.P. units per gram by the factor 1.36 indicated above, one obtains a potency for vitamin A alcohol of 4.749×10^6 U.S.P. units per gram. On the basis of these calculations the value of 4.30×10^6 U.S.P. units per gram of vitamin A alcohol as originally reported by Baxter and Robeson ('42) is perhaps explained. If the potency of 3.50×10^6 U.S. P. units per gram of vitamin A alcohol which has been reported more recently (Embree, '47; Hanze et al., '48) is based upon the present U.S.P. unit, it is in close agreement with the potency observed in this investigation. It is evident from the above studies on the relationships among the different vitamin A standards, and from stability and spectral absorption studies reported elsewhere of solutions

TABLE 3

Comparative potencies of the various vitamin A carriers, as measured by the spectrophotometric method and by the biological method of assay, and calculated conversion factors

ASSAY GROUP	SOURCE OF VITAMIN A	$E^{1\%}_{1\ cm}$ — 325 mμ [1]	CALCULATED POTENCY	BIOLOGICAL POTENCY FOUND IN TERMS OF [2]		CONVERSION FACTOR BASED UPON	
			$E^{1\%}_{1\ cm}$ — 325 mμ × 2000	U.S.P. Reference Cod Liver Oil no. 3	New U.S.P. Vitamin A Reference Standard	U.S.P. Reference Cod Liver Oil no. 3	New U.S.P. Vitamin A Reference Standard
				units/gm			
1	New U.S.P. Vitamin A Reference Standard	5.25	10,500	13,600	....	2590	....
2	U.S.P. Reference Cod Liver Oil no. 3	.817	1,634	....	1,250	....	1530
3	Crystalline vitamin A acetate	1520.0 [3]	3,040,000	4,141,000	3,045,000	2720	2000
4	Swordfish liver oil, domestic	80.6	161,600	177,500	130,500	2200	1620
5	Swordfish liver oil, imported	62.7	125,400	184,600	135,700	2940	2160
6	Skip Jack liver oil	20.8	41,600	43,500	31,980	2090	1540
7	Skip Jack liver oil, imported	25.1	50,200	56,000	41,170	2230	1640
8	Bluefin tuna liver oil	29.0	58,200	54,100	39,760	1870	1370
9	Bluefin tuna liver oil, imported	8.05	16,100	14,300	10,520	1780	1310
10	Totuava liver oil	28.6	57,200	45,450	33,420	1590	1170
11	Totuava liver oil, imported	74.7	149,400	144,600	106,300	1940	1420
12	Albacore liver oil, no. 1	35.5	71,000	108,300	79,600	3050	2240
13	Albacore liver oil, no. 2	7.69	15,380	16,620	12,220	2160	1590
14	Barracuda liver oil	42.5	85,000	57,700	42,420	1360	1000
15	Black cod liver oil	40.3	80,600	100,450	73,850	2490	1830
16	Bonita liver oil	31.4	62,800	78,780	57,930	2510	1850
17	Halibut liver oil	9.53	19,060	13,210	9,710	1390	1020
18	Jewfish liver oil	321.0	642,000	797,200	586,200	2480	1830
19	Ling cod liver oil	40.0	80,200	56,110	41,260	1400	1030
20	Mackerel liver oil	41.6	83,200	102,200	75,160	2460	1810
21	Menuke liver oil, imported	71.7	143,400	148,600	109,300	2070	1520
22	Sebastus Marinus liver oil	8.89	17,780	27,120	19,940	3050	2240
23	Spearfish liver oil, imported	51.0	102,000	102,200	75,130	2000	1470
24	Yellow Tail liver oil	20.1	40,200	43,780	32,190	2180	1600
25	Argentine shark oil	15.4	31,000	30,700	22,560	1990	1470
26	Dogfish liver oil	4.56	9,100	13,900	10,220	3050	2240
27	Blue shark oil	10.7	21,400	12,200	8,970	1140	840
28	Fortified cod liver oil	1.99	4,000	3,280	2,410	1650	1210
29	Soupfin shark oil	63.4	127,000	113,000	83,100	1780	1310
30	Mexican shark oil	17.4	35,000	33,800	24,840	1940	1430
Arithmetic mean of conversion factors for the fish oils						2100	1550
Logarithmic mean of conversion factors for the fish oils						2040	1500

[1] Nonsaponifiable fraction dissolved in isopropyl alcohol.

[2] Samples 1 to 24, inclusive were assayed simultaneous'y against the New U.S.P. Vitamin A Reference Standard and against U.S.P. Reference Cod Liver Oil no. 3. Samples 25 to 30, inclusive, were assayed simultaneously against U.S.P. Reference Cod Liver Oil no. 3, which in turn was subsequently assayed against the New U.S.P. Vitamin A Reference Standard.

[3] For the unsaponified cyrstalline vitamin A acetate.

similar to the present U.S.P. Vitamin A Standard (Guerrant et al., '48; Chilcote et al., '48a, '48b) that definite progress is being made in solving some of the problems encountered in vitamin A determinations.

The potency of the different fish oils is presented in table 3. Here it is to be noted that different samples of oil from the same species of fish frequently show wide differences in $E\,^{1\,cm}_{1\%}$ —325 mμ value, as well as in biological potency. Whether these differences are due to the feeding habits of the respective fish or to previous handling of the oils cannot be determined from the available information.

Concerning conversion factors

Conversion factors for the vitamin A standards and for the various fish oils were calculated by dividing the potency found by bioassay by the $E\,^{1\%}_{1\,cm}$ — 325 mμ value obtained for the unsaponifiable fraction of the oil when dissolved in isopropanol. Furthermore, average conversion factors were calculated arithmetically and also logarithmically, as suggested by Gridgeman ('44). The relatively high conversion factors based upon U.S.P. Reference Cod Liver Oil no. 3 as the standard and the low conversion factors based on the present U.S.P. Vitamin A Reference Standard (essentially the same as the International Standard), are merely reflections of the relative biological potencies of the two standards. Inasmuch as these standards were originally supposed to contain units of equal biological value the wide discrepancy in conversion factors based on them again suggests that U.S.P. Reference Cod Liver Oil no. 3 had lost about one-fourth of its biological potency before being used.

An examination of the conversion factors presented in table 3 indicates that a single such factor is not applicable to all fish oils, a conclusion previously reached by other investigators (Coy et al., '43; Zscheile and Henry, '44). This conclusion is also suggested by a summary of the various conversion factors presented by Gridgeman ('44). A satisfactory explanation

[3] For the unsaponified crystalline vitamin A acetate.

for the frequently observed variations in conversion factors for different vitamin A carriers has not been offered up to the present time. The inherent errors of biological assay may be contributing factors, but they certainly are not sufficient to account for wide variations, which range up to 250%. The presence or absence of varying amounts of materials in vitamin carriers which interfere with spectrophotometric measurement of vitamin A seems to offer a logical explanation for part of the variation among conversion factors. However, in these and in other studies neither saponification nor chromatography was found to be uniformly effective in reducing the relative variability of the calculated conversion factors, although in general both treatments resulted in higher factors for the treated oils than were obtained for the whole oils. Another possible explanation which is receiving considerable emphasis at the present time is based on the belief that spectrographic data are reasonably reliable but that the vitamin A present in various fish oils and vitamin carriers varies in its molecular structure and also in its availability to the test animal. Such an explanation seems to be based on logical premises; however, it is of interest to note that published data are not available to show that the biological potency of fish liver oils can be increased by saponification, a treatment which tends to convert all forms of the vitamin to the alcohol form. Furthermore, no published data are available to show that conversion factors calculated from biological data obtained by feeding the nonsaponifiable residues of fish liver oils are less variable than are the corresponding factors based on the biological assay of the whole oil.

Concerning the variabilities in the conversion factors reported in the present study, there appeared to be no indication of a consistent association of high or of low conversion factors with high or low biological potencies, nor was there any definite relationship between the L-values of the assay solutions and the respective conversion factors.

Methods which may be employed to increase the accuracy of the results obtained through spectrophotometric analysis

TABLE 4

Conversion factors for vitamin A acetate and for fish oils when calculated on the basis of the $E^{1\%}_{1\ cm}$ — 325 mμ of the whole oils and of the respective nonsaponifiable fractions and of the bone meal chromatographed residues

ASSAY GROUP	SOURCE OF VITAMIN A	BIOLOGICAL POTENCY OF WHOLE OIL	CONVERSION FACTOR BASED ON		
			Whole oil	Nonsaponifiable fraction	Bone meal chromatographed fraction
		U.S.P. units/gm			
1	New U.S.P. Vitamin A Reference Standard	10,000	.....	.....	.....
2	U.S.P. Reference Cod Liver Oil no. 3	1,250	1390	1530	1540
3	Crystalline vitamin A acetate	3,045,000	.....	2000 [1]	.....
4	Swordfish liver oil, domestic	130,500	1590	1620	1640
5	Swordfish liver oil, imported	135,700	2080	2160	2190
6	Skip Jack liver oil	31,980	1440	1540	1560
7	Skip Jack liver oil, imported	41,170	1630	1640	1760
8	Bluefin tuna liver oil	39,760	1290	1370	1570
9	Bluefin tuna liver oil, imported	10,520	910	1310	1480
10	Totuava liver oil	33,420	1120	1170	1220
11	Totuava liver oil, imported	106,300	1400	1420	1600
12	Albacore liver oil, no. 1	79,600	2220	2240	2410
13	Albacore liver oil, no. 2	12,220	1410	1590	1620
14	Barracuda liver oil	42,420	980	1000	1870
15	Black cod liver oil	73,850	1830	1830	1900
16	Bonita liver oil	57,930	1840	1850	1920
17	Halibut liver oil	9,710	940	1020	1110
18	Jewfish liver oil	586,200	1820	1830	2180
19	Ling cod liver oil	41,260	1000	1030	1050
20	Mackerel liver oil	75,160	1720	1810	1880
21	Menuke oil, imported	109,300	1460	1520	1620
22	Sebastus Marinus liver oil	19,940	2120	2240	2300
23	Spearfish liver oil, imported	75,130	1400	1470	1740
24	Yellow Tail liver oil	32,190	1560	1600	1630
25	Argentine shark oil	22,560	1470	1470	1540
26	Dogfish liver oil	10,220	2170	2240	2350
27	Blue shark oil	8,970	810	840	1030
28	Fortified cod liver oil	2,410	1210	1210	1310
29	Soupfin shark oil	83,100	1330	1310	1390
30	Mexican shark oil	24,840	1390	1430	1550
Arithmetic mean conversion factor for the fish oils			1480	1550	1680
Logarithmic mean conversion factor for the fish oils			1430	1500	1620

[1] Based on the $E^{1\%}_{1\ cm}$ — 325 mμ of the crystalline ester.

for vitamin A have been suggested in recent years (Morton and Stubbs, '46, '47, 48; Müller and Reinert, '46). The latter workers claim that by applying an aluminum oxide adsorption technique previous to spectrophotometric measurements, conversion factors of 1700 are obtained; whereas, if the oil is not first chromatographed by this technique, conversion factors below 1000 result. This is partially borne out by the data presented in table 4, where the mean conversion factor for the bone meal chromatographed residues (Glover, Goodwin and Morton, '47) from the various vitamin A carriers used in the present studies was found to be higher than the mean conversion factor for the whole oils or for the nonsaponifiable residues.

Morton and Stubbs ('48) have reported on a technique whereby spectrophotometric measurements are made at different wave lengths and certain corrections are made for irrelevant absorption. These authors claim that by this procedure a conversion factor of 1800 is obtained, whereas a factor of 1600 is obtained when the calculations are based on the gross absorption measurements. It appears, however, that further investigations of this type must be carried out before the biological potencies of all fish oils can be evaluated by physical means.

SUMMARY

A comparison has been made of the extinction coefficients and the relative biological potencies of U.S.P. Reference Cod Liver Oil no. 3, the new Vitamin A Reference Standard, crystalline vitamin A acetate, and the International Standard. In addition, 27 representative fish oils have been assayed biologically against these standards and conversion factors calculated. The results obtained in this investigation may be summarized as follows:

1. The extinction coefficients of the vitamin A standards employed were found to be:

   (a) U.S.P. Reference Cod Liver Oil no. 3: whole oil — 0.914; unsaponifiable fraction — 0.817.

(b) New U.S.P. Vitamin A Reference Standard: whole oil — 5.24; unsaponifiable fraction — 5.25.

(c) Crystalline vitamin A acetate — 1520.

(d) International Standard (β-carotene) — 2300.

2. Stability studies conducted with vitamin A and carotene solutions prepared for feeding purposes showed these sources of vitamin A to be stable over a two-week feeding period and during 120-day storage at 5°C., provided the solutions were protected against atmospheric oxidation.

3. A comparison of the relative biological potencies of the Vitamin A Standards indicated that the present U.S.P. unit and the International unit may be considered equal. The U.S.P. unit as represented by the new U.S.P. Vitamin A Reference Standard was found to be 1.36 times as potent biologically as the U.S.P. unit represented by U.S.P. Reference Cod Liver Oil no. 3. The potency of the crystalline vitamin A acetate was found to be 3.045×10^6 U.S.P. units per gram.

4. The conversion factors calculated for 27 representative fish oils (nonsaponifiable fraction) in terms of the present U.S.P. Vitamin A Reference Standard ranged from 840 to 2240, with a logarithmic mean of 1500. Conversion factors for the same oils calculated in terms of U.S.P. Reference Cod Liver Oil no. 3 ranged from 1140 to 3050, with a logarithmic mean of 2040. These mean values (1500 and 2040) indicate the extent of the decrease in potency of the cod liver oil standard, and may suggest a partial explanation for the existing confusion regarding conversion factors. The variability of the conversion factors for the fish oils used in the present study indicates that no one single conversion factor will be readily applicable to all fish oils.

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COMPARISON OF THE ASCORBIC ACID CONTENT OF PLASMA AND OF WHOLE BLOOD AS CRITERIA OF NUTRITIONAL STATUS

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TWO FIGURES

(Received for publication September 13, 1948)

As is discussed in their recent papers, Roe and coworkers ('46, '47) found plasma ascorbic acid values in both man and the guinea pig higher than those of whole blood when whole blood values were 0.6 mg % or above, whereas at lower whole blood levels plasma values were lower than those of whole blood. The above authors interpreted these findings as indicating that a whole blood ascorbic acid level of 0.6 mg % is an adequate level and that an adequate intake of the vitamin is an amount which will maintain the whole blood level at or above this concentration. This interpretation would be very significant if further investigation should show that plasma ascorbic acid levels are consistently higher than those of whole blood at some specific level.

The purpose of the present investigation was to compare the ascorbic acid concentration in the whole blood with that in plasma with regard to (1) levels at which the values of plasma exceed those of whole blood and (2) the variability of ascorbic acid values under conditions of depletion and repletion.

[1] Submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Home Economics, University of Chicago.

[2] The study of urinary excretion of ascorbic acid has been submitted by Miss Attaya in partial fulfillment of the requirements for the Master of Science degree, Department of Home Economics, Louisiana State University.

METHOD

Eight healthy young people were placed on a controlled diet and frequent determinations were made of whole blood and plasma ascorbic acid levels and of the 24-hour urinary excretion of the vitamin. The subjects were 7 students at Louisiana State University and one faculty member. All were living the usual life of science students engaged in laboratory and class activities and none was inclined to take part in strenuous athletics. Table 1 shows the sex, height, weight and age of each subject.

TABLE 1

Description of subjects and ascorbic acid dosage

CATEGORY OF INTEREST	SUBJECT							
	1	2	3	4	5	6	7	8
Sex	F	F	F	M	M	M	M	M
Height (in.)	67	67	67½	71½	69	67½	71	65½
Weight (lb.)	124	135	120	180	162	130	170	135
Age (years)	19	22	25	28	35	23	23	22
Ascorbic acid dosage								
Preliminary period (mg)	100	100	100 200	200	200	100	100	100
Preliminary period (days)	11	11	8 3	11	5	11	11	11
Depletion period, basal diet only (days)	25	25	25	25	25	25	25	25
Repletion period, basal diet and ascorbic acid								
100 mg morning (days)	21	21	21	21	28	28	28	28
100 mg in morning and 100 mg at supper (days)	8	8	8	8	14	14	14	14
200 mg at supper (days)	4	4	4	4	..	..	..	..
400 mg test dose at supper (days)	..	..	..	..	1	1	1	1
Basal diet (days)	2	2	2	2	2	2	2	2

Preliminary period

During a preliminary period all subjects were given an ascorbic acid supplement in addition to their usual diet in order to start the experimental period with all individuals in a good state of nutrition with regard to this vitamin.

Depletion period

After this preliminary period the subjects were placed on a basal diet which was prepared and served in the laboratory. The diet was designed to satisfy metabolic needs in all respects except as regards ascorbic acid. The ascorbic acid content of this diet had been determined repeatedly in connection with a previous study by the senior author (Hollinger, '48) and found to average 15 mg daily with little variation.

Repletion period

Following 25 days of depletion a daily supplement of 100 mg of ascorbic acid was added to the basal diet. This supplement was taken in the morning after blood samples were obtained. Subjects 1, 2, 3 and 4 continued on this regime for three weeks, after which an additional 100 mg of ascorbic acid was provided at the evening meal for 8 days. For another 4 days the entire 200 mg supplement was taken at the evening meal. The supplement was then discontinued while determinations of whole blood and plasma ascorbic acid and the urinary excretion of the vitamin were continued two days longer.

Because the remaining 4 subjects had shown a slower rise in whole blood and plasma ascorbic acid and urinary excretion values, they continued for 28 days on the 100 mg supplement, after which an additional 100 mg of ascorbic acid were taken at the evening meal for 14 days. This regime was followed by the administration of a 400 mg test dose. Determinations of whole blood and plasma ascorbic acid were made for three successive days, and of urinary excretion for two successive days, immediately following the ingestion of the 400 mg test

dose, during which time no further supplements were taken. All changes in the intake of ascorbic acid were made on the day just preceding the taking of a blood sample.

The ascorbic acid supplements (100 and 200 mg) were selected because they were presumed to be large enough to produce rapid rises in blood values and to give ample opportunity for comparing whole blood and plasma values under conditions of rapid repletion.

During the depletion and repletion periods whole blood and plasma ascorbic acid determinations were made on Monday, Tuesday, Thursday and Friday of each week, in order to compare values for consecutive days. Blood samples were drawn immediately after breakfast, which contained no foods having appreciable amounts of ascorbic acid.

About 12 ml of blood were taken from the arm by venous puncture and transferred to a centrifuge tube containing two or three drops of 20% potassium oxalate solution to prevent coagulation. After thorough mixing and the pipetting off of an aliquot of whole blood, the remainder was centrifuged for about 45 minutes to separate cells from plasma.

Twenty-four hour urine collections were made on 5 days each week, beginning on Monday morning and ending Saturday morning. Urine was collected in brown jars containing about 2 gm of crystalline oxalic acid and 2 ml of toluene.

The method of Roe and Kuether ('43) was followed in detail for the determination of ascorbic acid in whole blood, plasma and urine.

RESULTS

Whole blood and plasma ascorbic acid values in milligrams per cent, together with the urinary excretion values in milligrams, are presented in figures 1 and 2.

When the data from the 283 comparable samples of whole blood and plasma are examined as a whole it is evident that, with the exception of 15 samples, plasma values were lower than those of whole blood. During the entire depletion period only one sample was obtained in which the plasma value was higher than that of whole blood (fig. 1 B), although in 59

samples whole blood ascorbic acid values were found to be 0.6 mg % or above.

During the repletion period plasma values were lower than those of whole blood, with 14 exceptions. In 4 samples obtained from subjects 1 and 2 early in the repletion period the ascorbic acid value of the plasma slightly exceeded that of the whole blood (fig. 1, A and B). The remaining 10 samples in which the plasma ascorbic acid value exceeded that of whole blood were obtained from subjects 1, 3, 5 and 8 near the end of the repletion period while the 200 mg supplement was being taken, and, in the cases of subjects 5 and 8, after the 400 mg test dose. These latter 10 samples were obtained at a time when the subjects were saturated, as evidenced by the excretion of one-third or more of their ascorbic acid intake. In these subjects the excess of ascorbic acid in the plasma was not consistent but occurred in one or two samples, followed by samplings in which plasma values were lower than those of whole blood. In subjects 4, 6, and 7 plasma values were lower than those of whole blood in every sample obtained during both depletion and repletion, although these subjects maintained whole blood levels of 0.6 mg % or above during the last two to three weeks of depletion. These three subjects were saturated, as evidenced by the excretion of one-third or more of their ascorbic acid intake, for two weeks before the supplement was withdrawn (fig. 1 D, fig. 2, F and G). Subject 2 excreted 100 mg or more during the entire period on the 200 mg supplement but showed no plasma ascorbic acid value higher than that of whole blood during the repletion period.

The results of the present study show no evidence of a definite ascorbic acid level at which the plasma value consistently exceeds that of whole blood. This observation is in direct contrast to the findings of Roe and coworkers ('46, 47), who reported that in most cases where whole blood ascorbic acid values were 0.6 mg % or above, the plasma concentration was higher than that of whole blood. The evidence appears rather to confirm the conclusions of Heineman ('41)

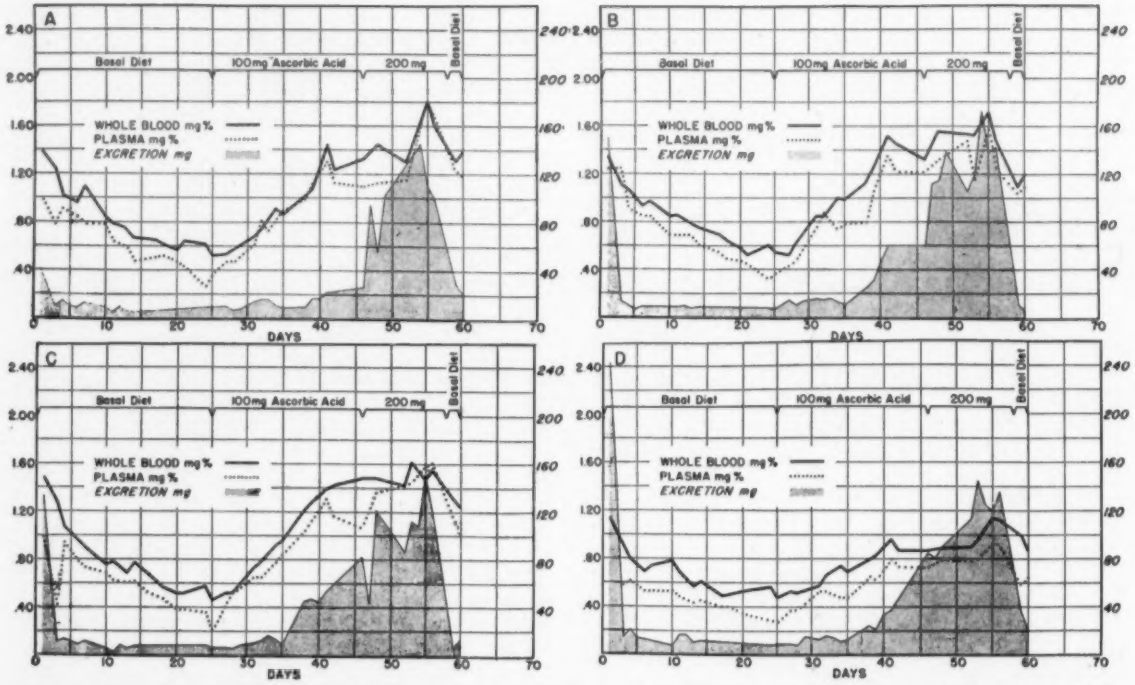

Fig. 1 A, B, C and D refer to subjects 1, 2, 3 and 4, respectively. Left ordinate, scale for plasma and whole blood. Right ordinate, scale for 24-hour urinary excretion.

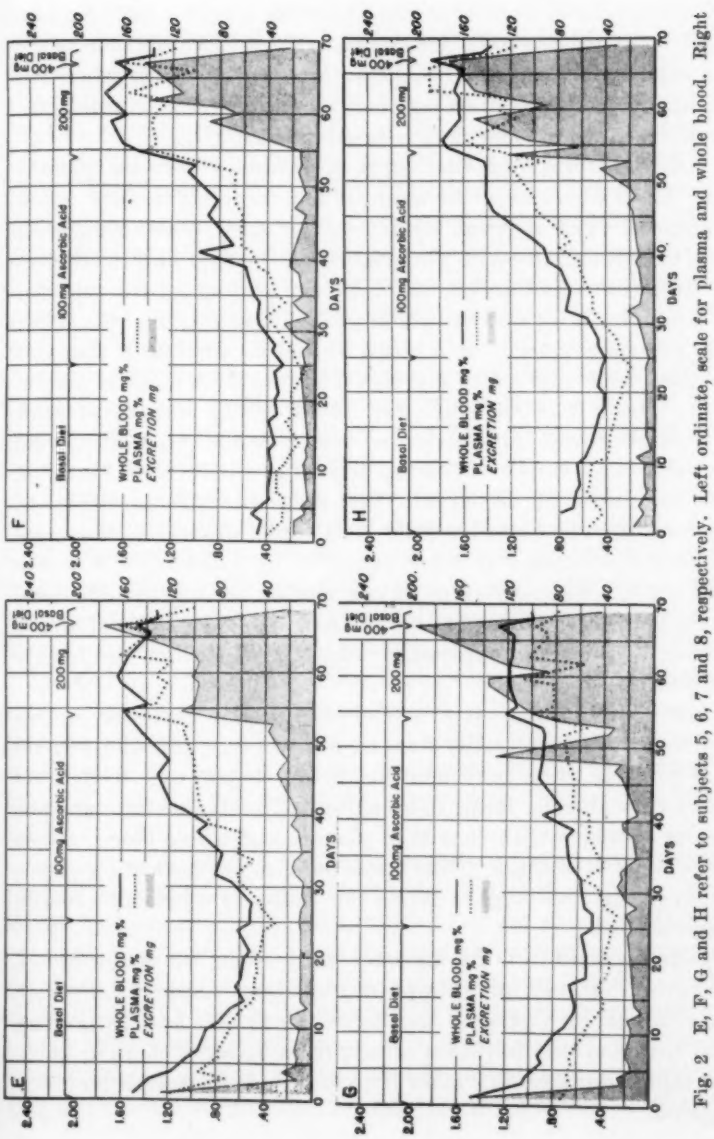

Fig. 2 E, F, G and H refer to subjects 5, 6, 7 and 8, respectively. Left ordinate, scale for plasma and whole blood. Right ordinate, scale for 24-hour urinary excretion.

and of Kyhos et al. ('45), who observed ascorbic acid concentrations of plasma to be greater than those of whole blood only transiently, following absorption of ascorbic acid, when the exchange of the vitamin between serum and cells caused fluctuations in their respective concentrations of it.

It should be noted that large differences between plasma and whole blood ascorbic acid values occurred in only a few instances. The average difference between plasma ascorbic acid values and those of whole blood on the first and third days of depletion was 0.38 mg %, while the average difference for the remainder of the depletion period was 0.15 mg %, showing a tendency for whole blood values to approach those of plasma somewhat more closely after the first days of depletion. There was no consistent trend either toward smaller or toward larger differences between plasma and whole blood ascorbic acid values following the large differences found at the beginning of depletion. The average difference for all samples during both depletion and repletion was 0.18 mg %, while the average difference reported by Lubschez ('45) was 0.14 mg %. The coefficient of correlation among the 283 comparable plasma and whole blood ascorbic acid values obtained during the entire experiment was 0.936, which is highly significant.

Estimates of the error variance, i.e., the variance among values for the same individual on a given day, for whole blood and plasma were very similar, being 0.0102 and 0.0091 respectively (Lucas, '48). In addition, estimates of the variance among subjects with regard to plasma and whole blood values were nearly the same. Thus these data do not indicate a choice between the two values from the point of view of random variabilities.

Determination of the ascorbic acid levels of whole blood, however, has the advantage of including white cells, which were found by Crandon and associates ('40) and by Pijoan and Lozner ('44) to retain an appreciable ascorbic acid value for a considerable time after the vitamin disappeared from the plasma.

The urinary excretion of ascorbic acid, as might be predicted, followed a different pattern from that of the whole blood and plasma values. In contrast to the gradual fall of whole blood and plasma ascorbic acid values, the average 24-hour ascorbic acid excretion of the 8 subjects fell abruptly from 108 mg on the last day of the preliminary period to 14 mg on the third day of the depletion period. Further decreases during the depletion period were small and irregular. Considerable individual variation was observed in the excretion of subjects having similar plasma and whole blood ascorbic acid values. At the beginning of repletion the urinary excretion rose slowly and irregularly at first, tending to show a sudden marked increase, which occurred in two subjects while taking the 100 mg and in the remainder while taking the 200 mg supplement. In those subjects who had shown a large increase in excretion on the 100 mg supplement, increase of the supplement did not cause a corresponding increase in excretion. Similarly, the 400 mg test dose given to subjects 5, 6, 7 and 8, following two weeks when 200 mg of ascorbic acid had been taken daily, was followed by increased retention. This increase in retention when intakes of ascorbic acid are increased has been reported previously by Johnson and Zilva ('34) and by Hamel ('37). The first 24-hour excretion of one-third or more of the intake coincided with plasma values of 0.78 to 1.59 mg % (figs. 1 and 2). When supplements were withdrawn at the end of the repletion period the urinary excretion for the two succeeding days again showed a marked decline.

It may be concluded that data based on the urinary excretion of ascorbic acid must be interpreted with caution. The large fluctuations in excretion values for the same individual on a constant low intake, and the slow and irregular response of these values to large increases in intake, leave serious doubts as to the reliability of a test of the habitual or "resting level" of the urinary excretion of ascorbic acid as a criterion of nutritional status. In the present investigation, however, large increases in excretion in response to an intake of 200 mg

or more of ascorbic acid coincided with plasma levels of 0.8 mg % or above, which is commonly accepted as being indicative of a satisfactory state of nutrition with regard to this vitamin.

CONCLUSIONS

1. It may be concluded that fasting plasma ascorbic acid values are in most instances lower than those of whole blood.
2. There is no level at which plasma ascorbic acid values are consistently higher than those of whole blood and which may be used as a criterion of blood cell saturation.
3. The random variabilities of plasma and of whole blood are very similar.
4. Urinary excretion of ascorbic acid is a poor indication of nutritional status with respect to this vitamin except when very high excretion values, indicating saturation, are obtained.

ACKNOWLEDGMENTS

The authors are indebted to Miss Margaret Green, formerly of the Department of Bacteriology, Louisiana State University, for obtaining all blood samples, and to the General Education Board for partial financial support.

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EFFECT OF ARSONIC ACID DERIVATIVES IN STIMULATING GROWTH OF CHICKENS

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TWO FIGURES

(Received for publication September 14, 1948)

Morehouse and Mayfield ('46), in reporting the effect of some aryl arsonic acids on experimental coccidiosis in chickens, stated that 3-nitro, 4-hydroxyphenyl arsonic acid had remarkable growth-stimulating properties for chickens and turkeys when given in the drinking water at 25% of the concentration necessary for coccidiosis control. The data on which this statement was based were not included in the article, but the authors kindly made available to us their unpublished material. The improvement in growth which they obtained in their experiments as the result of administering the above-mentioned compound was indeed remarkable. It seemed possible that this compound might be performing the same function as the unknown growth and hatchability factor which experiments in this laboratory have shown to be present in fish meal and in the feces of cows and chickens (Rubin and Bird, '46). Experiments were therefore planned to determine the optimum dietary level of 3-nitro, 4-hydroxyphenyl arsonic acid for stimulating the growth of chickens, to test the effectiveness in this respect of compounds chemically related to it, and to compare its effectiveness in several diets with the effectiveness of concentrates of the unknown factor.

METHODS AND RESULTS

Determination of optimum level

The formulas for the basal diets are given in table 1. Diet A was used in the two experiments designed to determine the optimum level of 3-nitro, 4-hydroxyphenyl arsonic acid for growth and the extent of the growth response that could be achieved at this level. In each experiment individual groups

TABLE 1

Composition of basal diets

INGREDIENTS	DIET A	DIET B	DIET C
	%	%	%
Yellow corn	31.0	38.0	23.0
Barley	10.0	20.0	...
Wheat	20.0	...	...
Alfalfa meal	5.0	3.0	3.0
Soybean meal	30.0	35.0	70.0
Butyl fermentation solubles (250 μg riboflavin per gram)	0.6	0.6	0.6
Steamed bonemeal	1.5	1.5	1.5
Limestone flour	1.0	1.0	1.0
Manganized salt [1]	0.5	0.7	0.7
Iodized salt [2]	0.2	..	..
Vitamin A and D oil [3]	0.2	0.2	0.2
	100.0	100.0	100.0
Nicotinic acid (mg/100 gm)	...	1.0	1.0

[1] Ninety-six per cent NaCl and 4% $MnSO_4 \cdot 4H_2O$.

[2] NaCl 100 parts, KI 0.3, $Na_2S_2O_3 \cdot 5H_2O$ 0.4, $CaCO_3$ 3.3, cornstarch 6.0.

[3] Four hundred A.O.A.C. units of vitamin D and 2000 I.U. of vitamin A per gram.

were fed diets which contained, respectively, 0, 0.0025, 0.005, and 0.01% of 3-nitro, 4-hydroxyphenyl arsonic acid and in one experiment an additional group received 0.02%. In each experiment one group received 5% of fish meal. Groups of 40 chicks were housed in the separate sections of a long brooder house. Each section contained an electrically heated hover and a steam radiator to provide supplementary heat. Wood shavings were used as litter. The chicks were three-

way crossbreds, being the progeny of New Hampshire males and Rhode Island Red X Barred Rock crossbred females. The dams of the chicks used in experiment 2 received different diets, some of which were adequate and some deficient with respect to the unknown factor required for growth and hatchability. To allow for the expected early mortality, lots of 50 day-old chicks were started on the experiment and the number reduced to 40 when the chicks were one week old by removing the smallest surviving chicks from each lot. The chicks in

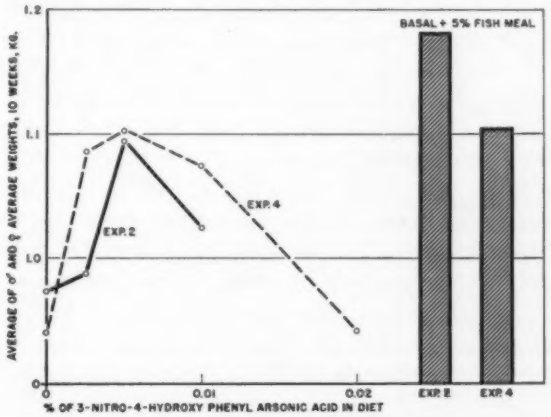

Fig. 1 Average weights of 10-week-old chickens fed 3-nitro, 4-hydroxyphenyl arsonic acid (at various levels) or 5% of fish meal as a supplement to diet A.

experiment 4 were all from dams that received diets adequate with respect to the unknown factor. The chickens were weighed at intervals of two weeks until they were 10 weeks old, at which time the experiments were terminated.

The results are given in figure 1. The optimum level of the arsonic acid derivative was 0.005% of the diet, and the groups fed this amount showed a substantially greater body weight than the basal groups at 10 weeks of age, the differences being 121 gm in experiment 2 and 163 gm in experiment 4. The response due to the arsonic acid derivative was com-

parable to that due to 5% of fish meal in experiment 4, but not in experiment 2.

Testing of related compounds

Diets A and B (table 1) were used in these experiments. The various compounds tested for their effect on growth are

TABLE 2

Average weights at 6 weeks of age of chickens fed various compounds

SUPPLEMENT TO BASAL DIET [1]	AVERAGE BODY WEIGHT AT 6 WEEKS IN EXPERIMENT NUMBER			
	5	6	7	8
	gm.	gm.	gm.	gm.
None	245	312	357	366
0.075% of acid ppt. of H_2O extract of cow manure	...	413	494	424
0.005% 3-nitro, 4-hydroxyphenyl arsonic acid	380	357	411	437
0.000375%-$Na_2HAsO_4 \cdot 7H_2O$	266	...	...	...
0.00075%-$Na_2HAsO_4 \cdot 7H_2O$	262	...	...	...
0.0015%-$Na_2HAsO_4 \cdot 7H_2O$	243	...	...	...
0.003%-$Na_2HAsO_4 \cdot 7H_2O$	246	...	...	...
0.005% phenyl arsonic acid	...	416 [2]	434	368
0.005% *p*-hydroxyphenyl arsonic acid	...	373 [3]	425	395
0.005% *p*-chlorophenyl arsonic acid	...	327	...	...
0.005% *m*-nitrophenyl arsonic acid	...	...	398	399
0.005% 3-nitrosalicylic acid	...	...	375	365
0.005% *m*-nitrobenzenesulfonic acid	...	...	331	360
0.005% sodium *p*-phenolsulfonate	...	...	338	320
0.005% salicylic acid	...	...	335	369
Least significant difference, odds of 19:1	50	62	64	50

[1] Basal diet A was used in experiment 5 and basal diet B in the other experiments.

[2] Level fed was 0.004 rather than 0.005%.

[3] Level fed was 0.0045 rather than 0.005%.

listed in table 2. In experiment 5, sodium arsenate was compared with 3-nitro, 4-hydroxyphenyl arsonic acid as a supplement to diet A. In the next three experiments diet B, the same diet used in the studies on the unknown growth factor, was used and the diet of the positive control groups was sup-

plemented with 0.075% of the acid precipitate of the water extract of cow manure to supply the unknown factor (Rubin and Bird, '46). It is not believed that the differences between diets A and B are of any significance with respect to these experiments.

The chicks used in experiments 5 and 6 were three-way crossbreds of the same derivation as those described above. The chicks used in experiment 7 were the crossbred progeny of Barred Rock males and Rhode Island Red females. The dams of the chicks used in these three experiments received a diet deficient in the unknown growth factor. Rhode Island Red chicks from hens fed a "complete" diet were used in experiment 8. Each group contained 25 chicks in experiment 5 and 12 in each of the other experiments. The chicks in experiment 5 were fed the experimental diets from hatching time to the age of 6 weeks; those in the other experiments, from the ages of two to 6 weeks. When the chicks were distributed among the experimental groups at two weeks of age, the very light and very heavy chicks were discarded and the groups equalized with respect to average weight. All groups were housed in steel batteries with screen floors.

The results are summarized in table 2. The data were analyzed by the method of Titus and Hammond ('35). The chickens fed 3-nitro, 4-hydroxyphenyl arsonic acid weighed more in all cases than did those fed the basal diet. The differences were statistically significant in experiments 5 and 8 and fell short of statistical significance in experiments 6 and 7. Phenylarsonic acid produced a marked stimulus to growth in experiments 6 and 7 and no stimulus in experiment 8. The chicks fed *p*-hydroxyphenyl arsonic acid were heavier than those fed the basal diet in all three experiments. In one experiment the result was clearly significant; in the other two it fell short, though only by one gm in experiment 6. In two experiments there appeared to be some growth response due to *m*-nitrophenyl arsonic acid but in neither case was the difference statistically significant. Growth was not stimulated by sodium arsenate, *p*-chlorophenyl arsonic acid, 3-nitrosal-

icylic acid, *m*-nitrobenzenesulfonic acid, sodium *p*-phenolsulfonate, or salicylic acid.

Comparative growth response of chickens fed the 3-nitro, 4-hydroxyphenyl arsonic acid, the unknown growth factor and methionine with different diets

As shown in figure 1 and in table 2, 3-nitro, 4 hydroxyphenyl arsonic acid stimulated growth when added to diets deficient in the unknown growth factor. In some experiments it was as effective as a concentrate of this factor; in others it was not.

TABLE 3

Average weights at 6 weeks of age of chickens fed various supplements with a diet containing 70% of soybean meal

GROUP NO.	SUPPLEMENT TO DIET C	AVERAGE WEIGHTS AT 6 WEEKS	
		By groups	By diets
		gm	*gm*
1	None	295	311
2	None	326	...
3	5% dried cow manure	467	453
4	5% dried cow manure	441	...
5	0.4% DL-methionine	360	338
6	0.4% DL-methionine	315	...
7	0.005% 3-nitro, 4-hydroxyphenyl arsonic acid	385	397
8	Same as 7	408	...

In an attempt to determine if the two supplements served the same function they were added to a diet containing 35% of raw soybeans rather than processed meal and to a diet containing 70% of processed soybean meal. It has been shown (Rubin and Bird, '47) that the latter diet is very effectively supplemented by the unknown growth factor but not by DL-methionine, and that the unknown factor is less effective in supplementing raw than processed soybean meal.

An experiment was performed in which diet C (table 1), containing 70% of soybean meal, was fed to 8 lots of chickens

with the supplements listed in table 3. The chickens were three-way crossbreds of the same derivation as those described above. The groups were housed in the separate pens of a brooder house with shavings for litter, and were fed the experimental diets from the ages of one to 6 weeks.

The results summarized in table 3 show that the arsonic acid derivative was effective when fed with 70% of soybean meal but less effective than was 5% of dried cow manure,

TABLE 4

Effect of type of soybean product and of supplements on average weights of chickens at 6 weeks of age

MODIFICATION OF DIET B		AVERAGE BODY WEIGHTS OF CHICKENS AT 6 WEEKS IN EXPERIMENT NUMBER		
Soybean product	Supplement [1]	9	10	11
		gm	*gm*	*gm*
Raw soybeans	None	221	318	289
Raw soybeans	A.A.	289	375	327
Raw soybeans	A.P.	324	390	314
Raw soybeans	M.	347	422	...
Raw soybeans	A.A. + A.P.	387	412	368
Raw soybeans	A.A. + M.	398	444	389
Raw soybeans	A.P. + M.	370	434	401
Raw soybeans	A.A. + A.P. + M.	411	462	411
Heated soybean meal	A.P.	...	...	449

[1] A.A. = 3-nitro, 4-hydroxyphenyl arsonic acid; 0.005% in experiments 9 and 10, 0.01% in experiment 11.

A.P. = acid precipitate of water extract of cow manure; 0.075% in experiments 9 and 10; 0.15% in experiment 11.

M. = DL-methionine; 0.4%.

which supplied the unknown growth factor. As reported previously DL-methionine was ineffective in promoting growth.

Since varying the dietary level of soybean meal did not lead to any definite conclusion as to whether the effects of the growth factor and the arsonic acid were identical, three experiments were performed in which these supplements and methionine were fed alone and in various combinations in a diet containing 35% of raw soybeans instead of heated soybean

meal. Groups of 12 crossbred chicks (Barred Rock X Rhode Island Red) were used in all three experiments. During the first two weeks of life they were fed diet B containing heated soybean meal without supplement. At two weeks of age they were divided into groups according to the procedure described above. From that time until they were 6 weeks of age they were fed the experimental diets, which were modifications of diet B. The nature of the dietary modifications and the average weights of the chickens are summarized in table 4.

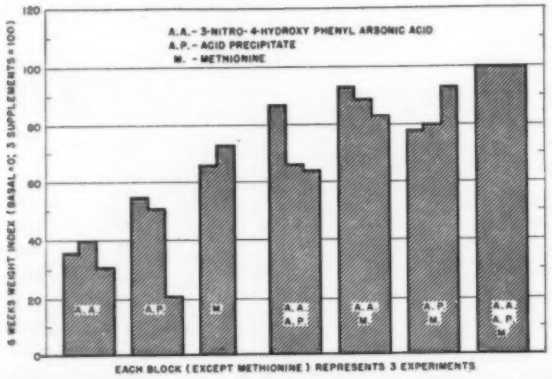

Fig. 2 Effects of 3-nitro, 4-hydroxyphenyl arsonic acid, the acid precipitate of a water extract of cow manure, and DL-methionine, fed separately and in various combinations, on the growth of chickens up to the 6th week.

To facilitate comparison of the three experiments the same data are summarized in figure 2. In each experiment the weight of the group receiving all three supplements was taken as 100 and the weight of the basal group as 0, and a value was calculated for each of the other groups to show its position between the basal group and the group receiving the supplements. It was obvious (1) that the methionine was the most effective of the single supplements; (2) that any pair of supplements was superior to any supplement alone (except that in experiment 10 methionine alone was superior to the other two supplements in combination); and (3) that the three

supplements together were superior to any pair. These results indicate that the three materials were mutually supplementary.

As shown in the footnote to table 4, the levels of the arsonic acid derivative and the acid precipitate were doubled in experiment 11. This was done to make sure that the apparent effect of the supplements observed in experiments 9 and 10 was not simply additive. The higher levels of the single supplements were not more effective in promoting growth than the lower levels. This result rules out the possibility of an additive effect.

A positive control group which received commercial heated soybean meal supplemented with the unknown factor was included in experiment 11. As shown in table 4, the diet containing raw soybeans plus all three supplements was still slightly inferior to the positive control diet.

DISCUSSION

The results of these studies confirm the report of Morehouse and Mayfield ('46) that 3-nitro, 4-hydroxyphenyl arsonic acid stimulates the growth of chickens when fed with certain diets. The optimum level of this compound for stimulating growth was 0.005% of the diet. The chickens fed 0.01% were definitely inferior to those fed 0.005% and those receiving 0.02% were no better than the basal group, showing that any attempt to make practical use of this compound would necessitate very careful regulation of the level fed.

Of the compounds tested only phenyl arsonic acid and its nitro- and hydroxy-derivatives were effective in promoting growth. The presence of the nitro- and hydroxy-groups was not essential. On the contrary, their presence may have resulted in reduced effectiveness although this was not definitely established by these experiments.

Tangl ('42) reported that the growth of chickens was stimulated by feeding cupri-sulfarsenite at a level supplying 0.035 mg of arsenic per bird per day. The composition of the basal diet was not stated. If there is a common denomina-

tor that accounts for Tangl's results and those reported in this paper, it will necessarily be a simple inorganic arsenic compound, especially since the compound fed by Tangl was more effective per unit weight of arsenic than the arsonic acid derivatives. The 3-nitro, 4-hydroxyphenyl arsonic acid fed as 0.005% of the diet supplied approximately 0.5 mg of arsenic per chicken per day. The 4 levels of sodium arsenate (table 2) supplied, respectively, 0.03, 0.06, 0.12 and 0.24 mg of arsenic per chicken per day.

Although sodium arsenate was not effective, it is possible that inorganic arsenic may exert a stimulating effect when liberated slowly within the organism. The metabolism of the arsenic compounds used in these experiments was not investigated, but it was found by Crawford and Levvy ('47) that after intravenous injection of phenyl arsonic acid into rabbits 90% of the urine arsenic was in the form of phenyl arsonic acid and 10% in the form of phenyl arsenious acid; no arsenious or arsenic acid was found. Apparently phenyl arsonic acid does not readily liberate inorganic arsenic compounds in the rabbit.

According to Gordon and Quastel ('48), organic trivalent arsenoxides combine reversibly with the thiol groups of enzymes, thus acting as inhibitors to the thiol enzymes. Although pentavalent organic arsenic compounds did not directly affect such enzymes, they were reduced by thiol compounds to trivalent arsenoxides which did combine with the enzymes. Enzyme inhibition is not a likely explanation for a growth-stimulating effect, but the possibility that this effect may involve reaction with some biologically important thiol compound is an interesting one.

The experiments with the diet containing raw soybeans indicate that the effect of the arsonic acid derivatives is distinct from the effects of the unknown growth factor and methionine, since these three materials supplemented each other. Since the arsonic acid derivative was more effective when fed with heated soybean meal than when fed with raw soy-

beans, its effect is not believed to be related to the trypsin inhibitor found in raw soybeans.

SUMMARY

The growth of chickens fed diets high in soybean meal and deficient in the unknown dietary factor found in fish meal and in cow manure was improved by the addition to the diet of 0.005% of 3-nitro, 4-hydroxyphenyl arsonic acid. The effect of higher or lower levels was less favorable. This compound did not function as a substitute either for the unknown factor or for methionine. On the contrary, with diets containing raw soybeans the arsonic acid derivative, the unknown factor, and methionine were mutually supplementary. The arsonic acid derivative was effective when fed with 35% or with 70% of commercially heated soybean meal, but less effective than the unknown factor. Other compounds comparable in their activity to 3-nitro, 4-hydroxyphenyl arsonic acid were phenyl arsonic acid and *p*-hydroxyphenyl arsonic acid. *M*-nitrophenyl arsonic acid also showed some growth-promoting activity. The following were tested and found inactive in this respect: *p*-chlorophenyl arsonic acid, sodium arsenate, *m*-nitrobenzenesulfonic acid, salicylic acid, 3-nitrosalicylic acid, and sodium *p*-phenolsulfonate.

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ADDENDUM

After this paper was submitted, evidence was obtained which indicated that the "unknown factor" mentioned here is vitamin B_{12}. (Lillie, R. J., C. A. Denton, and H. R. Bird, '48. Relation of vitamin B_{12} to the growth factor present in cow manure. J. Biol. Chem., *176:* 1477.)

PANTOTHENC ACID AND CONJUGATION REACTIONS IN VIVO

I. THE EFFECT OF PANTOTHENIC ACID AND THIAMINE DEFICIENCIES ON THE ABILITY OF THE RAT TO ACETYLATE SULFANILAMIDE [1]

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ONE FIGURE

(Received for publication September 14, 1948)

The finding that pantothenic acid is a constituent of Coenzyme A (Lipmann et al., '47; Kaplan and Lipmann, '47) which functions in the acetylation of aromatic amines and choline *in vitro* led naturally to a study of the *in vivo* role of this vitamin in various acetylation reactions. Martin and Rennebaum ('43) had previously reported, on the basis of observations of blood levels, that pantothenic acid- and pyridoxine-deficient rats acetylated sulfanilamide at a normal rate, whereas thiamine- and riboflavin-deficient rats were defective in this respect.

While the present study was underway Riggs and Hegsted ('48) presented evidence for an *in vivo* role of pantothenic acid in the acetylation of *p*-aminobenzoic acid. This report on the acetylation of sulfanilamide confirms their observations and presents additional evidence on this subject.

[1] This work was supported by a grant from the National Vitamin Foundation. Preliminary studies were carried out with the assistance of a grant (RG 312c) from the U. S. Public Health Service.

METHODS

Pantothenic acid deficiency was induced by feeding a diet (Diet III-P) with the following percentage composition: Casein (vitamin-free) 20; cerelose 66; corn oil 10; salts (GBI salt mixture no. 2) 4; and with the following vitamins per 100 gm of the above basal mixture: percomorph oil, three drops; thiamine, 1 mg; riboflavin, 1 mg; pyridoxine, 1 mg; niacin, 2 mg; folic acid (Folvite), 0.20 mg; biotin, 0.002 mg; and choline, 100 mg. The control diet (Diet III) contained in addition 5 mg of calcium pantothenate per 100 gm of basal diet. The thiamine-deficient diet was similar to diet III except for the omission of thiamine.

Urine was collected over a 22-hour period from individual rats in metabolism cages. Sulfanilamide was injected intraperitoneally as an 0.8% water solution and analysis for the free and acetylated forms was performed by the method of Bratton and Marshall ('39). The percentage of the total excreted sulfanilamide which was acetylated was calculated and served as the basis for comparison in the following experiments.

EXPERIMENTAL PROCEDURES AND RESULTS

1. Sulfanilamide dosage and acetylation rate

Without prior knowledge of the sensitivity of the acetylation mechanisms in relation to dosage and to possible effects of pantothenic acid deficiency, it was important to determine the optimum conditions for estimating the effects of the deficiency. The administration of too much sulfanilamide would flood the organism with the aromatic amine, so that the percentage acetylated by the rat would be low; too little of the drug might not tax the acetylation mechanism to the point where the effect of a deficiency would be manifest.

The percentage acetylation by both control and pantothenic acid-deficient rats was determined when different amounts of sulfanilamide were injected at varying intervals. The acetylation with two, three or 4 hourly injections of 16 mg each of

sulfanilamide as an 0.8% solution did not differ appreciably, indicating that the acetylation mechanism was not overtaxed. With 5 hourly injections there was a definite decrease in acetylation by both the deficient and the control animals. Nevertheless consistent results were obtained, but at lower percentage levels. While any one of these dosage procedures would be useful in testing acetylation, three hourly injections seem most suitable for two reasons, namely (a) insuring the excretion of an adequate amount of sulfanilamide for analytical purposes without the danger of flooding the organism unduly, and (b) avoiding an unnecessarily large number of injections.

It was noted that normal young male rats weighing about 75 gm or less acetylated much smaller percentages than when older and heavier. This makes it impossible to get a satisfactory base line before placing very young rats on a deficient diet.

2. Pantothenic acid deficiency and acetylation

Several experiments were carried out with male rats to determine the acetylation ability with progressive deficiency and recovery.

In figure 1 is shown the course of events when 9 male rats, initially weighing about 70 gm, were fed the deficient diet, while each of 7 paired litter-mate rats were fed a control diet in amounts similar to or below that of the deficient litter mate. All of these rats received hourly intraperitoneal injections of 2 ml of an 0.8% sulfanilamide solution for the first 5 hours of urine collection. Within three weeks significant differences in the percentage of sulfanilamide acetylated were noted between control and deficient animals.

The ability of the deficient rats to recover their power to acetylate following calcium pantothenate administration is also shown in figure 1. On the day indicated by the first arrow, three of the 8 remaining deficient rats were injected with 250 μg calcium pantothenate and the acetylation test carried out the same day. Thereafter these three animals received

the control diet containing 5 mg of calcium pantothenate per 100 gm of diet. A similar procedure was followed with another three deficient rats on the 65th day. The pantothenate injections had no immediate effect but maintenance on the control diet brought acetylation levels back to normal.

In another experiment pantothenic acid deficiency was induced in somewhat older male animals and maintained for approximately twice as long. The control animals were maintained at approximately the same weights as the deficient

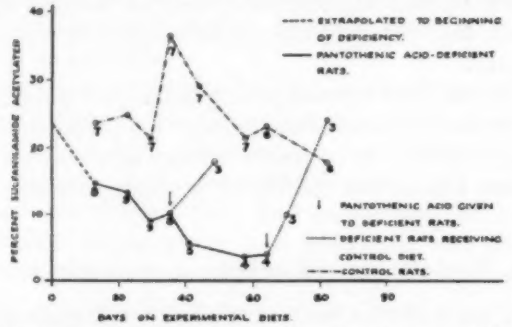
EXTRAPOLATED TO BEGINNING OF DEFICIENCY.
PANTOTHENIC ACID-DEFICIENT RATS.
PANTOTHENIC ACID GIVEN TO DEFICIENT RATS.
DEFICIENT RATS RECEIVING CONTROL DIET.
CONTROL RATS.
PERCENT SULFANILAMIDE ACETYLATED
DAYS ON EXPERIMENTAL DIETS.

Fig. 1 Effect of pantothenic acid deficiency in rats on acetylation of sulfanilamide. Numbers on the curves refer to the number of animals on which averages were taken. The first analyses were made after 8 days on the experimental diets. Five hourly injections, each of 16 mg sulfanilamide, were made at the beginning of each 22-hour urine collection.

rats by restriction of food intake. Data on acetylation percentages of these control and deficient animals are given in table 1. The data in the first column (—4 days) were obtained to check the uniformity of acetylation of all test animals while on the control diet, and were obtained with two injections (at zero and two hours) of 16 mg sulfanilamide each. All other data were obtained with three hourly injections at zero, one, and two hours of the urinary collection period; 24, 16, and 16 mg of sulfanilamide being given, respectively. The acetylation levels differ from those in figure 1 primarily because of the different amounts of sulfanilamide injected in the two series.

Both groups had similar levels of acetylation in the pre-deficiency period (—4 days), the mean differences between them being insignificant ($P = .08$ by the "t" test). As the deficiency progressed, marked decreases occurred in the

TABLE 1

Acetylation of sulfanilamide excreted by pantothenic acid-deficient and control rats
All values are in percentages.

DEFICIENT RAT NO.	DAY OF EXPERIMENT							
	— 4 [1]	+ 70	+ 79	+ 87	+ 94	+ 98 [4]	+ 105 [5]	+ 115 [6]
40D	40.0	15.3	16.8 [2]	14.1	16.2	19.0	18.3	29.5
41D	34.0	14.5	15.9 [2]	15.3	15.4	12.3	13.1	26.4
42D	36.4	16.3	15.7 [2]	10.0	12.3	9.5	10.4	25.2
43D	36.0	23.6	18.9 [3]	21.0	22.5	17.0	18.2	...
44D	40.3	16.2	14.1 [3]	16.1	16.0	16.2	15.3	28.6
45D	44.6	25.0	21.5 [3]	...	19.1	16.5	18.1	29.8
46D	28.4	15.2	12.4 [3]	10.8	14.2	13.4	14.5	27.6
48D	38.2	18.2	14.9 [3]	11.5	11.2	11.7	13.0	23.6
Average	37.2	18.0	16.3	14.1	15.9	14.5	15.1	27.2
CONTROL RAT NO.								
40C	48.5	39.6	32.5 [2]	32.8	32.2	28.9	31.6	29.0
41C	47.3	35.4	31.7 [2]	29.4	28.6	31.2	30.4	29.6
42C	38.2	35.9	32.1 [2]	34.2	33.4	31.7	33.2	29.0
43C	39.3	31.8	33.1 [3]	27.9	50.0	32.4	32.2	...
44C	37.4	34.0	29.4 [3]	25.8	28.7	29.4	28.4	33.0
45C	39.3	29.7	37.0 [3]	30.2	30.1	...	29.1	33.9
46C	41.4	33.0	29.2 [3]	35.6	32.2	28.6	27.8	27.7
47C	35.2	35.6	32.1 [3]	29.0	26.5	30.5	34.3	...
48C	33.8	31.2	28.8 [3]	...	33.0	24.1	29.6	31.8
Average	40.0	34.0	31.8	30.6	32.7	29.6	30.7	30.6

[1] On day — 4 all rats were tested for acetylation ability while on control diets, receiving only two injections of sulfanilamide (16 mg each at zero and two hours). The data in the following columns were obtained in acetylation tests with three injections of 16 mg of sulfanilamide at zero, one, and two hours.

[2] Rats received three successive daily injections of 0.2 ml of adrenal cortex extract (Upjohn) before acetylation test.

[3] Rats received one injection of 0.2 ml of adrenal cortex extract (Upjohn) several hours before acetylation test.

[4] Rats received an injection of 100 μg calcium pantothenate several hours before acetylation test.

[5] Rats received 4 injections of 100 μg calcium pantothenate each, spaced evenly during the week preceding acetylation test.

[6] Rats on control diet for one week preceding acetylation test.

acetylation percentages of the deficient group. After 70 days of deficiency, the average of the deficient animals was 53% that of the controls; this difference is highly significant ($P < .001$). As the deficiency period continued there was not much change in the acetylation percentage.

Corresponding to the results obtained in the initial experiment, 4 injections of calcium pantothenate (100 μg each) once every other day for one week had no appreciable effect on the deficient rats (105th day), but ingestion of the control diet for one week caused a marked increase in acetylation percentage (115th day).

None of the deficient animals exhibited severe symptoms; weight loss was gradual but porphyrin-like incrustations about the face were common. There was no close correlation between the extent of weight loss and the acetylation percentage.

Because of the relation of pantothenic acid to the structural integrity and function of the adrenal gland (Daft et al., '40; Ralli and Graef, '45; Deane and McKibbin, '46), the effect of adrenal cortical extract on acetylation was tested. Ten deficient and 10 control rats received subcutaneous injections of 0.2 ml extract of the adrenal cortex [2] on three successive days. The usual sulfonamide test and urine collection started 4 hours after the third administration. Another 4 deficient and 4 control rats received a single injection (0.2 ml) of the hormone preparation 4 hours before the beginning of the acetylation test. The particular batch of extract used was assayed in our laboratory for unbound pantothenic acid by the method of Skeggs and Wright ('44) and found to contain an insignificant amount (0.024 μg/ml). As is indicated in table 1 the extract had no significant effect on acetylation (79th day).

3. Thiamine deficiency and acetylation

Despite the food restriction of the control rats in the experiments described above, their physical appearance was much superior to that of the deficient animals. The question

[2] Upjohn's.

presented itself of whether the decreased acetylation of the deficient animals was due to general debility rather than to a specific lack of pantothenic acid. To help answer this question, and at the same time to provide a comparison with the findings of Martin and Rennebaum ('43), acetylation tests were performed on thiamine-deficient rats. In table 2 are given the averages of the percentage acetylation of sulfanilamide (5 hourly injections of 16 mg each) by thiamine-deficient male rats at various intervals and of their litter-mate controls kept

TABLE 2

Effect of thiamine deficiency on ability of the rat to acetylate sulfanilamide

TIME ON EXPERIMENTAL DIETS	AMOUNT OF SULFANILAMIDE ACETYLATED BY	
	Deficient rats [1]	Control rats [1]
	%	%
Control period	17.8	16.6
9 days	21.2	24.1
15 days	24.1	16.4
22 days	16.7	25.5
33 days	19.0	20.2
43 days	25.0	20.4
Average	20.6	20.5

[1] Average of 4 to 6 rats. All animals received intraperitoneal injections of 16 mg of sulfanilamide (0.8% solution) at zero, one, two, three and 4 hours of the 22-hour urine collection period.

at approximately the same weights by restricted feeding. During the last two weeks of the experiment it was necessary to give occasional injections of small amounts of thiamine to the deficient animals to keep them alive. Despite the acute deficiency, there was no significant difference in acetylation percentages between the two groups. This experiment was repeated with smaller amounts of sulfanilamide (24, 16, and 16 mg at zero, one, and two hours) and again no significant differences were noted between control and deficient male rats.

DISCUSSION

The findings reported here on the effect of pantothenic acid deficiency on the ability of rats to acetylate sulfanilamide confirm the *in vitro* studies of Lipmann et al. ('47) on the acetylation of sulfanilamide and the *in vivo* studies of Riggs and Hegsted ('48) on the decreased acetylation of *p*-aminobenzoic acid in pantothenic-deficient rats.

Our observation that thiamine deficiency exerted no influence on the ability of male rats to acetylate sulfanilamide does not support the finding of Martin and Rennebaum ('43) that thiamine deficiency in rats decreased the proportion of acetylated sulfanilamide in the blood. Nor is it in accord with the reports of Kinnunen ('46) and Suomalainen and Kinnunen ('46) that rabbits on a diet composed chiefly of oats and hay excreted a larger proportion of ingested sulfapyridine in the acetylated form following thiamine administration. Incidentally, no evidence was given by these workers that the rabbits on the oats-hay diet were deficient.

Failure to obtain an immediate acetylation response to injected calcium pantothenate (100 or 250 μg) is in keeping with the results of Riggs and Hegsted ('48), who found that while 1 mg of the vitamin restored acetylation of *p*-aminobenzoic acid to normal, 500 μg and 200 μg resulted in inconsistent and incomplete increases. It is noteworthy, however, that 4 injections of 100 μg each over a period of a week did not cause any significant response in our rats.

Quantitative data on the relationships among pantothenic acid intake, tissue Coenzyme A content, and acetylation rate would appear desirable for a variety of reasons, including the possibility of utilizing the acetylation reaction as a simple biochemical assessment of pantothenic acid status.

SUMMARY

1. Rats maintained on a pantothenic acid-deficient diet excreted much smaller percentages of test doses of sulfanilamide in the acetylated form than did control animals.

2. Adrenal cortical extract had no influence on the reduced ability of pantothenic acid-deficient rats to acetylate sulfanilamide.

3. Thiamine deficiency did not affect the ability of male rats to acetylate sulfanilamide as indicated by urinary excretion values.

ACKNOWLEDGMENT

Acknowledgment is made to Mr. Martin Sass and Mr. Amerigo Gambro for assistance in some of the experiments.

We are indebted to Merck and Company for many of the vitamins used, to Lederle Laboratories for the Folvite, and to Mead Johnson and Company for the percomorph oil.

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THE EFFECT OF THE LEVEL OF DIETARY CALCIUM AND MAGNESIUM ON THE DIGESTIBILITY OF FATTY ACIDS, SIMPLE TRIGLYCERIDES, AND SOME NATURAL AND HYDROGENATED FATS [1, 2, 3]

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ONE FIGURE

(Received for publication September 22, 1948)

Most natural fats and oils melting below 50°C. have long been recognized as being almost completely digestible. In a summary of previously reported tests on man, Deuel ('48) has noted that of 34 vegetable fats melting below 50°C., only two had coefficients of digestibility below 95. These were avocado oil (87.8%) and tea seed oil (91.2%). In the case of animal fats, 18 different varieties melting under 50°C. were found to be digestible to the extent of 93% or more in man.

There are a number of factors which alter the digestibility of fats. The most important of these is the melting point. Fats

[1] These data are from a thesis presented by Amber L. S. Cheng to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation.

[2] Aided by a grant from Swift and Company.

[3] Paper no. 188 from the Department of Biochemistry and Nutrition, University of Southern California.

melting above the critical level of 50°C. have been shown to have considerably reduced digestibilities. Thus, in man, mutton fat (m.p., 50°C.) has been reported to have a coefficient of digestibility of 88 (Langworthy and Holmes, '15), oleostearin (m.p., 50°C.) of 80.1 (Holmes, '19), and deer fat (m.p., 51.4°C.) of 81.7 (Deuel and Holmes, '22). Moreover, straight hydrogenated and blended hydrogenated vegetable oils melting above 50° have been found by Deuel and Holmes ('21, '22) to have lowered coefficients of digestibility. These workers ('21) have suggested that an inverse relationship exists between the absorbability of fats and their melting points.

However, Lyman ('17) suggested that the melting point is not the only factor in determining digestibility but that the fatty acid makeup of the ester is also an important consideration. He based his argument on the fact that tripalmitin, which has a m.p. of 56°C., is utilized to the extent of 95% in the dog, while Arnschink (1890) had earlier reported coefficients of digestibility of 9 to 14 for tristearin (m.p., 71°C.) in the same species. Hoagland and Snider ('43) have obtained similar differences in tests on rats, the coefficients of digestibility being 84 to 82 and 8 to 6 for tripalmitin and tristearin, respectively, when the triglycerides constituted 5 or 10% of an olive oil solution which in turn was fed at a 5% level. Mattil and Higgins ('45) have also demonstrated that some variation in digestibility obtains when comparable fatty acid mixtures are administered as components of a mixed triglyceride, as contrasted with their administration as mixtures of proportionate amounts of simple triglycerides. Thus, the average digestibility of distearomono-olein when fed at a 15% level was 66, while the coefficient of digestibility of a mixture of 10% tristearin and 5% triolein was found to be only 40.

Another factor of importance in determining the digestibility of high melting triglycerides is the nature of the other fat components. When tristearin is dissolved in olive oil (Hoagland and Snider, '43) or is present as a component of natural fats so that the melting point of the mixture is

below 50°C., a high digestibility obtains because of the aid of the solvent action of such fats with lower melting points as triolein. The presence of such emulsifying agents as lecithin also increases the utilization of fats with high melting points (Augur et al., '47).

Moreover, there is some evidence that the species of animal plays a role as a factor in digestibility. Guinea pigs have been shown by McCay and Paul ('38) to have lower capacities for absorption, not only of hydrogenated fats but also of such common vegetable oils as corn oil, in comparison with man. On the other hand, such other herbivora as the rabbit and the sheep were found to digest common fats as well as man (Paul and McCay, '42–'43). An interesting difference between these species and man is the ready digestibility of castor oil in the former group. Although the rat and dog in general have responses to fat comparable to man's, it has recently been shown that considerable amounts of rapeseed oil escape absorption in the rat (Deuel, Cheng and Morehouse, '48) in contrast to its complete digestibility in man (Holmes, '18). The high digestibility of rapeseed oil in man has recently been confirmed in this laboratory.[4]

One important factor, which causes an alteration in fat absorption and which has been investigated only to a slight extent, is the effect of the presence of dietary calcium and magnesium. Givens and Mendel ('17) found that when the absorption of fats or fatty acids was poor, the excretion of fecal calcium was increased. In fact, calcium storage was decreased under such conditions even when the calcium intake was at a high level. Bosworth et al. ('18) concluded that the presence of ionized calcium in the intestine determines the extent of soap formation, although the amount ultimately excreted in the feces depends on the solubility of the soap formed. It was suggested that calcium oleate is more readily soluble in bile than calcium palmitate or calcium stearate; hence, it disappears from the intestine in larger quantities

[4] Unpublished experiments of H. J. Deuel, Jr., R. M. Johnson, C. M. Culbert and B. Thomas.

even in the presence of an excess of calcium. Boyd, Crum and Lyman ('32) reached similar conclusions. When a diet containing a relatively high calcium intake (60–70 mg per day) was fed to rats, the utilization of oleate, palmitate, and stearate soaps was found to be 90, 38, and 25%, respectively; on the other hand, when a relatively low calcium diet was employed (13.5–31.4 mg per day), the absorption of palmitate and stearate was increased to 65 and 45%, respectively. MacDougall ('38), on the other hand, demonstrated an antirachitic effect of lard or olive oil in a low calcium diet; this was explained as being due to an improved absorption of calcium as the oleate; in the absence of fat it was largely lost from the intestine in combination with phytin.

The present experiments were designed to determine the effect of the inclusion of calcium and magnesium in the diet on the excretion of soaps on diets relatively rich in fats. Studies have been made not only with some natural fats but also with a series of simple synthetic triglycerides and also several fatty acids.

EXPERIMENTAL AND RESULTS

The experimental procedure was similar to that used by Augur et al. ('47). Adult female rats weighing between 145 and 185 gm were fed ad libitum, over 7- to 10-day periods, diets containing the fats. In all cases a preliminary orientation period of two days was allowed, followed by an interval during which food consumption was measured and the feces collected for analysis. This latter period was 8 days in length where normal food fats were fed but was shortened to 5 or 6 days where animals were fed diets containing the synthetic triglycerides. Total glycerides and fatty acids were determined in the stools by diethyl ether extraction of the dried stool. Acidification of the fecal residue and a second extraction with ether yielded the portions present as soaps. The composition of the diets used is included in table 1.

Calculations for digestibility were made in the usual manner after correction for metabolic fat (Augur et al., '47). The

figures for metabolic fat were determined in tests where the rats had a similar diet but in which the total fat had been replaced by carbohydrate. The data giving the results of these experiments are summarized in table 2.

TABLE 1

Composition of the various diets used

INGREDIENT	DIET					
	A	B	C	D	E	F
Commercial casein (%)	18	18	18	18	18	18
Glucose (cerelose) (%)	59	61	61	61	74	76
Fat (%)	15	15	15	15	0	0
Yeast (Anheuser-Busch, G) (%)	1	1	1	1	1	1
Osborne-Mendel salt mixture (%)	7	5[1]	5[2]	5[3]	7	5
Ca content (mg/gm)	6.1	0	2.5	1.17	6.1	0
Mg content (mg/gm)	0.9	0	0.35	0.16	0.9	0

[1] Salts containing calcium and magnesium were omitted from the Osborne-Mendel salt mixture.

[2] Salts containing calcium and magnesium were added in half the amounts prescribed in the Osborne-Mendel salt mixture.

[3] Salts containing calcium and magnesium were added in one-quarter the amounts prescribed in the Osborne-Mendel salt mixture.

TABLE 2

Summary of control tests to determine metabolic fat of rats fed fat-free diets containing normal Ca and Mg content (diet E) and low in Ca and Mg (diet F)

DATA SUMMARIZED	DIET E (Fat-free containing 7% O.M.[1] salt mixture)	DIET F (Fat-free containing 5% O.M.[1] salt mixture with Ca and Mg omitted)
Number of rats	10	10
Average weight of rats (gm)	170	166
Average gain or loss (gm)	—4	—1
Average total weight of stools (gm)	3.83	1.53
Average fat excreted		
As neutral fat and fatty acid (mg)	119	149
As soaps (mg)	122	72
Total (mg)	241	221
Average fat/gm stool (mg)	65	148

[1] Osborne-Mendel.

TABLE 3

Summary of digestibility studies on some natural and hydrogenated fats fed with salts of calcium and magnesium (diets A) or without salts of these metals (diet B)

DATA SUMMARIZED	FAT FED											
	Crude rapeseed oil		Refined rapeseed oil		Bland lard		Hydrogenated lard		Blended lard [1]		Hydrogenated lard	
Melting point (°C.)	Liquid		Liquid		47.8		55.4		55.2		61.0	
Diet	A	B	A	B	A	B	A	B	A	B	A	B
Number of rats	10	5	10	5	5	5	5	5	5	5	5	5
Average weight of rats (gm)	175	175	160	165	167	168	177	172	177	168	174	169
Average gain or loss (gm)	+4	−4	−1	+1	+2	+2	0	−3	−1	−1	−5	−1
Average fat ingested (gm)	12.1	10.4	7.8	10.8	13.3	12.2	11.8	9.2	10.7	9.1	11.0	10.6
Average weight of stools (gm)	6.47	2.50	3.42	1.93	5.74	2.73	8.00	3.80	7.00	3.60	12.1	9.9
Average fat excreted												
As neutral fat and fatty acid (gm)	1.07	0.66	0.65	0.57	0.25	0.39	0.51	1.17	1.06	1.33	3.78	5.98
As soaps (gm)	2.12	0.51	0.99	0.39	1.16	0.54	4.67	1.40	2.97	1.16	6.03	1.99
Total (corrected) (gm)	2.77	0.81	1.42	0.66	1.03	0.51	4.66	2.01	3.57	1.75	9.03	6.50
Coefficient of digestibility [2]	77.2 ± 1.6	92.3 ± 1.4	82.5 ± 1.4	93.9 ± 0.6	92.4 ± 0.9	95.8 ± 0.6	58.0 ± 2.4	77.9 ± 4.8	66.2 ± 1.8	80.0 ± 1.9	17.3 ± 3.0	38.0 ± 3.6

[1] A mixture of one-third hydrogenated lard (m.p., 61°C.) and two-thirds prime steam lard (m.p., 37°C.).

[2] Including the standard error of the mean calculated by the formula $\sqrt{\frac{d^2}{n}}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

and "n" is the number of observations.

TABLE 4

Summary of digestibility studies on some synthetic simple triglycerides fed with calcium and magnesium salts (diets A, C, D) or without salts of these metals (diet B)

DATA SUMMARIZED	FAT FED									
	Trilaurin				Trimyristin		Tripalmitin		Tristearin	
Melting point (°C.)	49				56		66.5		70	
Diet	A	C	D	B	A	B	A	B	A	B
Number of rats	6	5	5	5	5	5	5	5	5	5
Average weight of rats (gm)	170	161	172	168	183	168	173	163	161	169
Average gain or loss (gm)	−4	+8	−5	0	+5	+2	−2	−1	0	−2
Average fat ingested (gm)	8.9	7.6	10.0	8.8	12.8	9.7	9.1	9.8	9.5	9.0
Average weight of stools (gm)	4.80	1.98	2.47	1.55	10.53	4.43	11.67	10.39	12.11	10.56
Average fat excreted										
As neutral fat and fatty acids (gm)	0.25	0.23	0.38	0.16	1.12	1.49	6.40	7.51	7.50	7.97
As soap (gm)	2.72	0.83	0.79	0.29	7.46	1.33	2.27	1.27	1.90	0.88
Total (corrected) (gm)	2.61	0.94	1.01	0.22	7.90	2.26	7 92	7.25	8.61	7.28
Coefficient of digestibility[1]	70.5 ± 1.7	87.2 ± 4.0	89.5 ± 0.8	97.3 ± 3.7	37.7 ± 1.4	76 6 ± 8.1	12.8 ± 1.2	27.9 ± 1.5	10.6 ± 2.9	18.9 ± 2.4

[1] Including the standard error of the mean calculated as described in table 3, footnote 2.

TABLE 5

Summary of digestibility studies on palmitic acid, stearic acid and monostearin fed with calcium and magnesium salts (diet A) or without salts of these metals (diet B) or as soaps (diet G)

DATA SUMMARIZED	FAT FED							
	Palmitic acid			Stearic acid			Monostearin	
Melting point (°C.)	63			69			59.9	
Diet	A	B	G[1]	A	B	H[2]	A	B
Number of rats	5	5	5	5	5	4	5	5
Average weight of rats (gm)	160	162	172	165	158	173	158	145
Average gain or loss (gm)	+1	−1	−13	−3	0	−5	−10	−5
Average fat ingested (gm)	9.7	9.1	9.4	9.8	9.6	9.6	10.1	9.5
Average weight of stools (gm)	10.4	8.9	9.46	11.8	9.80	10.3	12.1	7.37
Average fat excreted								
As neutral fat and fatty acids (gm)	3.89	4.98	1.08	3.99	6.92	1.30	3.22	4.00
As soaps (gm)	4.60	2.29	7.43	5.53	2.78	8.09	5.55	1.84
Total (corrected) (gm)	7.81	5.87	7.89	8.45	8.15	8.73	8.06	4.75
Coefficient of digestibility[3]	19.8 ± 3.4	35.6 ± 1.9	16.7 ± 4.0	14.4 ± 3.1	15.8 ± 2.9	9.5 ± 0.6	20.7 ± 2.3	47.4 ± 6.7

[1] Contains 10.00 gm of calcium palmitate and 5.68 gm of palmitic acid per 100 gm food (equivalent to 14.95 gm of palmitic acid and 76 mg of calcium).

[2] Contains 10.47 gm of calcium stearate and 5.21 gm of stearic acid per 100 gm food (equivalent to 14.94 gm of stearic acid and 76 mg of calcium).

[3] Including standard error of mean calculated as in table 3, footnote 2.

It is surprising that, although the total level of metabolic fat is about the same on diets E and F, the quantity per gram of stool produced on the calcium-magnesium-low diet is more than double that obtained when the diet contains these salts; this is attributed to the marked reduction in the weight of the stools excreted in the former case. The value found for diet E (65 mg) corresponds quite well with that determined by Augur et al. ('47), which was 50.5 mg.

Experiments on the digestibility of rapeseed oil,[5] bland lard,[6] and several samples of hydrogenated lard are summarized in table 3, while table 4 records the results of the tests with synthetic triglycerides.[7] Data on the absorption of palmitic and stearic acids fed as such with or without the calcium-magnesium salts or as the calcium soap are given in table 5.

DISCUSSION

The data presented offer some indication that an inverse relationship exists within a certain range between digestibility and melting point. Thus, in the series of tests with simple triglycerides where calcium and magnesium were present, the coefficients of digestibility were as follows: Trilaurin (m.p., 47.8°C.) 77.2; trimyristin (m.p., 56°C.) 37.7; tripalmitin (m.p., 66.5°C.) 12.8; and tristearin (m.p., 70°C.) 10.6. The values for the samples of blended and hydrogenated lard show somewhat higher digestibility in the lower range of melting points but approach the low level in the sample with m.p. of 61°C.

[5] The rapeseed oil was obtained from the Pacific Vegetable Oil Company and was the same sample employed in earlier tests (Deuel, Cheng and Morehouse, '48). The various lard samples were furnished by Swift and Company and were some of the same samples used in the tests of Crockett and Deuel ('47). Synthetic triglycerides were Eastman products, while the monostearin was a commercial product furnished by Dr. H. W. Vahlteich of The Best Foods Corporation. The monostearin had a saponification number of 156.7 (theory 156.2). The calcium salts of palmitic and stearic acids were prepared from the corresponding fatty acids by slow precipitation with a dilute $CaCl_2$ solution, followed by filtration and thorough washing with acetone and drying under a vacuum at 50°C.

[6] See footnote 5.

[7] See footnote 5.

The results were as follows: Bland lard (m.p., 47.8°C.) 92.4; hydrogenated lard and blended lard (m.p., 55.4 and 55.2°C.), 58.0 and 66.2; and hydrogenated lard (m.p., 61.0°C.) 17.3. The results of the tests where the calcium and magnesium content was at a minimum show similar relationships. These results are evident from an inspection of figure 1, where digestibility is plotted against melting point.

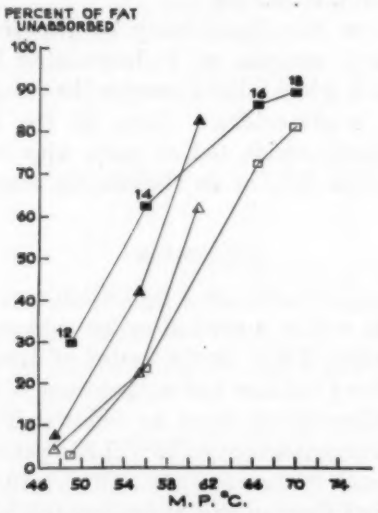

Fig. 1 The relationship of melting point to digestibility of simple triglycerides (■) and of samples of hydrogenated lard (▲), where calcium and magnesium were present in the diet. The results of tests with calcium-magnesium-low diets is represented in each case by similar characters which are not filled in. The numbers by each character indicate the number of carbon atoms in the fatty acid component of the simple triglycerides.

The present results do not confirm the earlier experiments of Lyman ('17) or Hoagland and Snider ('43), who reported that tripalmitin is digested to an amount greater than 80% in dogs and rats. In the present tests it was found that tripalmitin was absorbed only to the extent of 12.8% in rats, while this was increased to 27.9% when calcium and magnesium were removed from the diet. The low digestibility of tripalmitin in

our experiments is further confirmed by the results when the free acid or soap was fed; in these cases the coefficients of digestibility were 19.8 and 16.7, respectively. In the absence of calcium and magnesium this figure was increased only to 35.6. The reason for the discrepancies between our work and that of the earlier investigators is not immediately evident. In our tests no supplementary oil was given in the diet, while in those of Hoagland and Snider ('43) olive oil was present and must have dissolved the palmitic acid to a considerable extent.

The presence of calcium and magnesium in the diet does not change the digestibility of the natural fats with lower melting points. Thus the following high digestibilities have been reported by Crockett and Deuel ('47) for fats melting under 50°C. when fed with the high calcium-magnesium diet: Margarine (m.p., 34°C.) 97.0; commercial hydrogenated cottonseed oil [8] (m.p., 43°C.) 97.3; prime steam lard (m.p., 37°C.) 96.6; and bland lard (m.p., 48°C.) 94.3. On the other hand, the removal of calcium and magnesium from the diet markedly increases the digestibility of the fats with higher melting points although the effect becomes much less pronounced in the case of tristearin. In the calcium-magnesium diet, trilaurin is digested to the extent of 70.5%, while in the absence of these divalent ions the value rises to 97.3. Corresponding coefficients of digestibility for trimyristin under these divergent dietary conditions are 37.7 and 76.6, respectively, while with tristearin the values are 10.6 and 18.9. That this effect is related to a mass action phenomenon is indicated by the trilaurin tests, where the addition of smaller proportions of calcium and magnesium to the diet resulted in a lessened depressive action on digestibility.

When calcium and magnesium were omitted from the diet, a marked decrease in the proportion of fat excreted as soap occurred, while some increase in the neutral fat-fatty acid fraction usually resulted. This effect was most marked with trilaurin and trimyristin and in the cases of the natural and

[8] Crisco.

hydrogenated fats studied. The shift from the soap to the neutral fat-fatty acid fraction on the elimination of calcium and magnesium from the diet was much less pronounced in the case of the triglycerides; the bulk of the fat excreted even when calcium and magnesium were present in the diet was in the neutral fat-fatty acid fraction. That the low absorption of tripalmitin and tristearin is not solely the result of a failure in lipolysis is shown by the fact that the fatty acid content of the neutral fat-fatty acid fraction was 35% in the tripalmitin tests and 32% in the tristearin experiments. As the insolubility of the fatty acids increases, the tendency to form soaps is diminished. This may be the result of the extreme insolubility of the free acid plus a very low degree of ionization.

These data confirm the earlier report of Boyd, Crum and Lyman ('32) on the differential effect of the level of calcium intake in depressing the fat absorption as related to the fatty acid make-up of the diet. Conversely, the calcium intake level is of considerable importance in relationship to calcium retention as a function of the fat content of the diet. It is evident that a minimum calcium loss will occur when the dietary fats have low melting points; however, when the melting point of the ingested fat exceeds 50°C., one may expect a marked loss of calcium, proportional to the amount of such fat fed.

The elimination of calcium and magnesium from the diet markedly lowers the weight of the stools in all cases except those of tripalmitin and tristearin. In many cases stools from rats on calcium-magnesium-low diets weigh less than 50% of those where these ions are included in the rations.

SUMMARY

1. In a series of tests with simple triglycerides an inverse relationship obtained between the melting point and the coefficient of digestibility. A similar relationship was noted with hydrogenated lards of varying melting points.

2. Although the presence of calcium and magnesium in the diet does not influence the digestibility of low-melting fats, it markedly decreases that of higher-melting simple triglycerides and hydrogenated fats. The digestibilities with and without the divalent ions were as follows: Trilaurin, 70.5 and 97.3; trimyristin, 37.7 and 76.6; tripalmitin, 12.8 and 27.9; and tristearin, 10.6 and 18.9. With respect to several lards the corresponding coefficients of digestibility with and without the calcium-magnesium salts were as follows: Bland lard (m.p., 47.8°C.), 92.4 and 95.8; hydrogenated lard (m.p., 55.4°C.) 58.0 and 77.9; blended hydrogenated lard (m.p., 55.2°C.) 66.2 and 80.0; and hydrogenated lard (m.p., 61°C.) 17.3 and 38.0.

3. The absence of calcium and magnesium from the diet markedly increases the neutral fat-fatty acid fraction at the expense of the soap fraction. This effect is most marked with trilaurin, trimyristin, and the hydrogenated lard samples, and least with tripalmitin and tristearin. In the latter case the bulk of the excreted fat under either dietary regime is in the neutral fat-fatty acid fraction.

4. The effect of calcium and magnesium was a progressive one, being greater the larger the proportion of these salts in the diet.

5. In all cases except in the tests with tripalmitin and tristearin the elimination of calcium and magnesium from the diet resulted in a decrease in stool weight of 50% or more.

6. The removal of calcium and magnesium from the diet resulted in an increase in the digestibility of crude and refined rapeseed oil to 92 and 93%, respectively.

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EFFECT OF OATS, OAT PRODUCTS AND FAT ON THE INTESTINAL SYNTHESIS OF BIOTIN IN MATURE FOWL[1]

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TWELVE FIGURES

(Received for publication July 22, 1948)

The laying hen has been used as an experimental animal by Couch and associates ('48a) for studying the intestinal synthesis of biotin as reflected (1) by the biotin content of the egg and (2) by hatchability. These workers reported that dextrin favored the intestinal synthesis of this vitamin but that sucrose did not promote such synthesis, and further that lactose and dried whey did not stimulate the synthesis of biotin in the intestinal tract of the laying fowl. Congenital anomalies in the chick resulting from feeding hens a low biotin diet have been described by Cravens et al. ('44) and by Couch et al. ('48b).

The present investigation was initiated to determine the effect of oats, oat products and fat on the intestinal synthesis of biotin in the mature fowl. Hatchability of fertile eggs, incidence of congenital anomalies, and biotin content of the eggs were criteria used in evaluating the extent of intestinal synthesis and absorption of biotin.

[1] Supported in part by a grant from the Western Condensing Company, San Francisco, California. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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MATERIALS AND METHODS

The management and general experimental procedure was the same as that previously reported by Couch et al. ('48a).

The composition of the diets is shown in table 1. Crystalline B-vitamins with the exception of biotin and pteroylglutamic

TABLE 1

Composition of diets

	B31	B31A	B32	B32A	B34	B45	B45A	B46	B46A	B47	B48
	%	%	%	%	%	%	%	%	%	%	%
Sucrose	63	63	..	..	63	43	23	43	23	53	53
Dextrin	..	..	63	48	..	..	..	..	..	..	..
Ground whole oats	..	..	..	..	..	20	40	..	..	..	..
Ground oat groats	..	..	..	..	..	..	..	20	40	..	..
Ground oat hulls	..	..	..	..	..	..	..	..	..	10	10
Purified casein	18	18	18	18	18	18	18	18	18	18	18
Gelatin	5	5	5	5	5	5	5	5	5	5	5
Salts IV	5	5	5	5	5	5	5	5	5	5	5
Liver fraction "L"	4	4	4	4	4	4	4	4	4	4	4
Fish oil (3000A) (400D)	2	2	2	2	2	2	2	2	2	2	2
Soybean oil	3	3	3	18	3	3	3	3	3	3	7
Oyster shells	Ad libitum										
Vitamins and choline listed below added in mg per kg of diet											
Biotin	..	0.016	..	..	0.2	..	..	..	..	..	..
Thiamin HCl	4	4	4	4	4	4	4	4	4	4	4
Riboflavin	6	6	6	6	6	6	6	6	6	6	6
Ca pantothenate	15	15	15	15	15	15	15	15	15	15	15
Niacin	100	100	100	100	100	100	100	100	100	100	100
2-methyl-1,4-napthoquinone	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	4	4	4	4	4	4	4	4	4	4	4
Alpha-tocopherol	3	3	3	3	3	3	3	3	3	3	3
Choline	200	200	200	200	200	200	200	200	200	200	200

acid were added in amounts which were thought to meet the requirements of the birds. Liver fraction "L" was used as a source of the latter vitamin and also as a source of unidentified factors. Biotin was added to diets B31A and B34. Oyster shells and tap water were supplied ad libitum. The oat

products used were ground in a burr mill to a fineness which made for uniform distribution in the basal diet.

The pullets were fed an all-mash laying ration for 15 weeks prior to the start of the first experiment, during which period data were collected on egg production and hatchability. At the end of this pre-experimental period 16 birds were selected and divided into 4 groups of 4 birds each. The effect of 20% whole oats (B45), 20% oat groats (B46) and 10% oat hulls (B47) on the intestinal synthesis of biotin was studied by using diet B31 as a negative control and replacing an equivalent amount of sucrose with each of the above-mentioned ingredients. Two other groups of 4 hens, each of which had been used in an earlier experiment, were continued on diet B34 and the practical all-mash diet. The latter two groups served as positive controls in this experiment. The duration of the experimental period was 5 weeks.

Eight groups of 4 hens each were used in experiments 2 and 3; these were conducted concurrently. In experiment 2 the percentage of whole oats (diet B45A) and oat groats (diet B46A) was increased to 40; one group (diet B31A) was included in which 0.016 mg of crystalline biotin per kilogram of diet was added (equivalent to biotin in 40 parts of whole oats). Diet B47 (10% oat hulls) was repeated; and diet B31 was used as the negative control.

In experiment 3 the effect of fat on the intestinal synthesis of biotin was studied by using dextrin as the carbohydrate (B32) and increasing the fat from 5 to 20% (diet B32A). Further information was also obtained on the effect of fat through a comparison of diets B47 and B48. In diet B48 the soybean oil was increased to 7 parts; this makes diet B48 equal to diet B31 from a caloric standpoint without taking the oat hulls into account. Since experiments 2 and 3 were conducted concurrently and under the same conditions, data from hens fed diets B31 and B47 were included in both experiments. Hens fed diet B31 served as negative controls in both instances. In addition, hens fed diet B34 and the practical

all-mash diet (described above) were again used as positive controls. The duration of these experiments was also 5 weeks.

Data were also included from a 4th experiment in which one group of hens was fed diet B31 and another was fed diet B40, which was the same as B31 except that 5 parts of cellu-flour were substituted for an equivalent amount of sucrose.

Eggs were set aside for biotin determinations on the last two days of each week. A modification of the method of Wright and Skeggs ('44), in which turbidity was the criterion employed in measuring the growth of *Lactobacillus arabinosus,* was used in determining the biotin content of the eggs. The biotin content of the egg yolks and whites was expressed in millimicrograms per gram on a fresh weight basis. The biotin content of the oats and oat products was also determined.

During the course of the studies herein reported, biotin determinations were also made on feces from hens fed the practical all-mash diet.

It was found that symptoms of biotin deficiency could be ascertained in a 12-day-old embryo. All such embryos which died were used in determining the percentages of each on various diets that were affected with congenital anomalies traceable to a deficiency of biotin in the maternal diet.

RESULTS AND DISCUSSION

This investigation has shown that the feeding of ground whole oats promotes the intestinal synthesis of biotin when substituted for sucrose in a low biotin diet. When 20% whole oats was used (diet B45), the per cent of hatchability throughout the test was higher than that obtained with diet B31 (fig. 1). In the first experiment the hens fed diet B45 appeared to undergo an adjustment (possibly in the intestinal flora) during the first three weeks, at the end of which time the per cent of hatchability was 25. However, after such an adjustment, the hatchability of eggs from hens fed diet B45 increased to 75% at the end of the fifth week. The feeding of 20% whole oats likewise increased the biotin content of

the yolks and whites of eggs over that observed for eggs from hens fed diet B31 (figs. 3 and 4) and produced a smaller percentage of anomalies than in embryos and chicks from hens fed diet B31. The use of 20% oat groats (B46) in a low biotin diet also appeared to exert a favorable effect on the intestinal synthesis of biotin when hatchability (fig. 1), congenital deformities (fig. 2) and the biotin content of the eggs (figs. 3

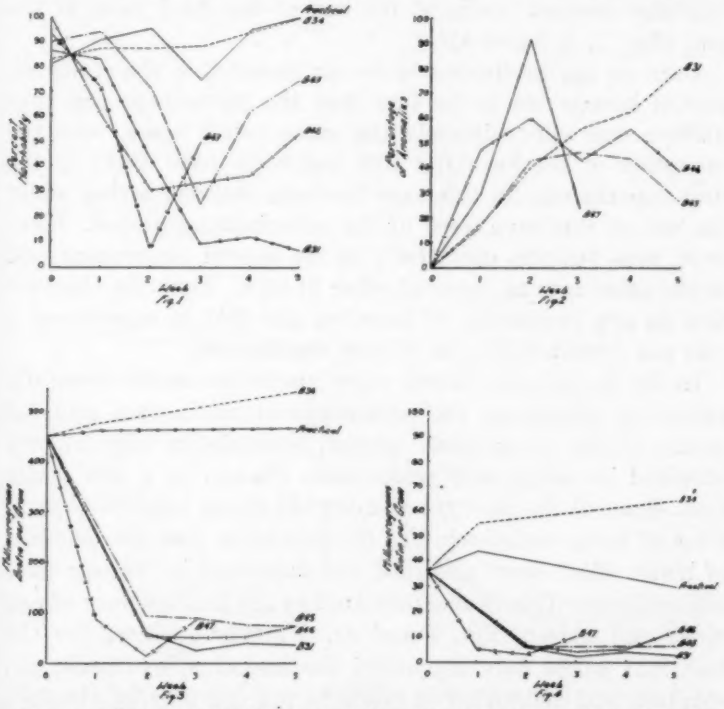

Fig. 1 Effect in hatchability of adding oats and oat products to a low biotin diet: diet B31, sucrose; diet B34, biotin; diet B45, 20% oats, diet B46, 20% oat groats; diet B47, 10% oat hulls.

Fig. 2 The incidence of congenital deformities as influenced by the addition of oats and oat products to a low biotin diet for breeding hens.

Fig. 3 Effect on the biotin content of egg yolk of adding oats and oat products to a low biotin diet.

Fig. 4 Effect on the biotin content of egg white of adding oats and oat products to a low biotin diet.

and 4) were considered, and a comparison was made with the results obtained with diet B31. The favorable effect on the intestinal synthesis of biotin appeared to be slightly greater in the case of the hens fed whole oats than in that of those fed oat groats. The results obtained through the feeding of oat hulls (B47) in the first experiment are somewhat erratic and inconclusive, due in part to the fact that the hens fed this diet stopped laying at the end of the third week of the test (figs. 1, 2, 3 and 4).

Data on egg production were not included in the study reported herein due to he fact that the percentage egg production was approximately the same in all cases, with the exception of the hens fed 10% oat hulls (diet B47) in the first experiment. In this case the hens stopped laying about the end of the third week of the experimental period. However, hens fed this diet (B47) in the second experiment laid at the same rate as those of other groups. Thus the observation on egg production of hens fed diet B47 in experiment 1 was not considered to be of any significance.

In the second experiment more conclusive results were obtained by increasing the percentage of whole oats and oat groats to 40. Apparently normal hatchability (fig. 5) was obtained by using 40% whole oats (B45A) in a low biotin diet. Some of the embryos and day-old chicks exhibited symptoms of biotin deficiency (fig. 6), indicating that the amounts of biotin which were absorbed and deposited in the egg were not optimum. This is also indicated by the biotin values of egg yolks and whites (figs. 7 and 8). Further evidence for the fact that whole oats supported the intestinal synthesis, absorption and deposition of biotin in the egg may be obtained by comparing the results on hatchability (fig. 5), congenital deformities (fig. 6), and the biotin content of the eggs (figs. 7 and 8) obtained by feeding 40% whole oats (B45), with those obtained by feeding diet B31A to which was added 0.016 mg of crystalline biotin (equivalent to that amount of the vitamin contributed by 40 parts of whole oats).

Forty per cent oat groats (diet B46A) favored the intestinal synthesis of biotin to a lesser extent than 40% whole oats (diet B45A, figs. 5, 6, 7, and 8). The fact that diet B46A supported the synthesis of the vitamin may be seen by comparing the per cent of hatchability (fig. 5), percentage of anomalies

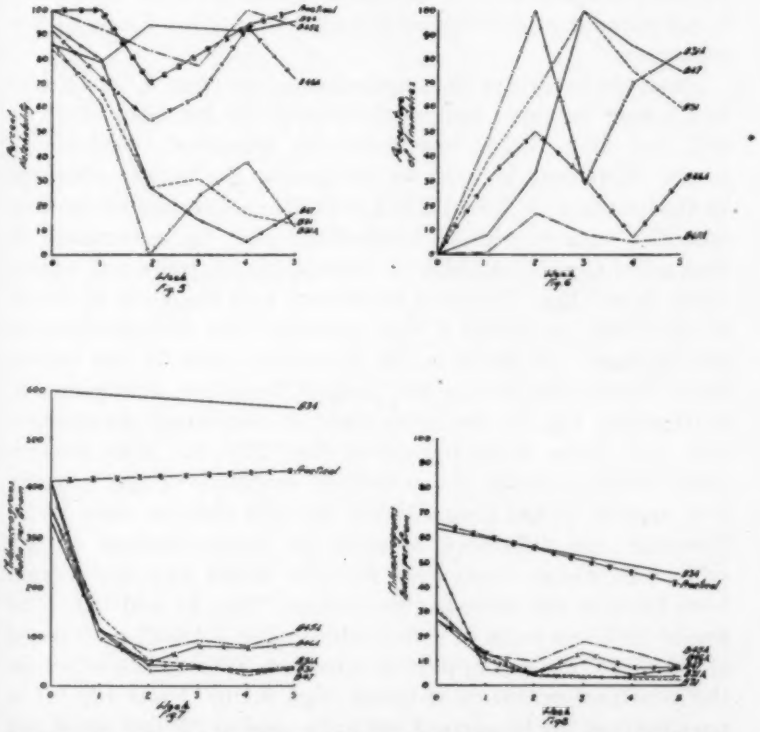

Fig. 5 Effect on hatchability of adding oats and oat products to a low biotin diet: diet B31, sucrose; diet B31A, 0.016 mg biotin per kilogram; diet B34, 0.2 mg biotin per kilogram; diet B45A, 40% oats; diet B46A, 40% oat groats; diet B47, 10% oat hulls.

Fig. 6 The incidence of congenital deformities as influenced by the addition of oats and oat products to a low biotin diet for breeding hens.

Fig. 7 Effect on the biotin content of egg yolk of adding oats and oat products to a low biotin diet.

Fig. 8 Effect on the biotin content of egg white of adding oats and oat products to a low protein diet.

(fig. 6), and biotin content of the egg yolks and whites (figs. 7 and 8) which resulted from feeding hens diet B46A with the data obtained when hens were fed diet B31.

The use of 10% oat hulls (diet B47) in a low biotin diet did not favor intestinal synthesis of biotin as far as hatchability (fig. 5), incidence of congenital deformities (fig. 6) or the biotin content of the egg yolks and whites (figs. 7 and 8) are concerned.

From the results of the third experiment (figs. 9, 10, 11, and 12) it may be noted that dextrin with 5% fat (diet B32) or 20% fat (diet B32A) supported the intestinal synthesis of biotin. This may be seen by comparing the results obtained by feeding diets B32 and B32A with those obtained by feeding diet B31 with respect to hatchability (fig. 9), percentage of anomalies (fig. 10) and biotin content of egg yolks and whites (figs. 11 and 12). This is in agreement with the work of Couch et al. ('48a), in which it was reported that dextrin favored the synthesis of biotin in the intestinal tract of the laying fowl. When the results are judged from the standpoint of hatchability (fig. 9) and percentage of congenital deformities (fig. 10), there is an indication that 20% fat with dextrin (diet B32A) favored the intestinal synthesis of the vitamin to a greater extent than did 5% fat with dextrin (diet B32). However, the difference between the biotin content of egg yolks and whites from hens fed diet B32A and those from hens fed diet B32 were not so striking (figs. 11 and 12). The use of 10% oat hulls in a diet with either 5 (B47) or 9 parts of fat (B48) did not appear to have any appreciable effect on the intestinal synthesis of biotin (figs. 9, 10, 11 and 12). It is possible that the 10 parts of oat hulls used in the diet could not overcome the effect of the principal carbohydrate (sucrose) used in diets B47 and B48, which does not support the synthesis of the vitamin in the intestinal tract of the fowl. Davis and Briggs ('47) reported that the addition of 5, 10 and 15% cellulose to a purified chick diet resulted in a significant increase in growth. However, these workers used "cerelose" as the source of carbohydrate. Five per cent cellu-flour failed

to have any effect on the intestinal synthesis of biotin in the 4th experiment of the present study. However, again it should be pointed out that sucrose was the carbohydrate, and it does not favor the synthesis of biotin in the alimentary tract of the laying fowl. It was observed in the present investigation that whole oats favored the intestinal synthesis of biotin in

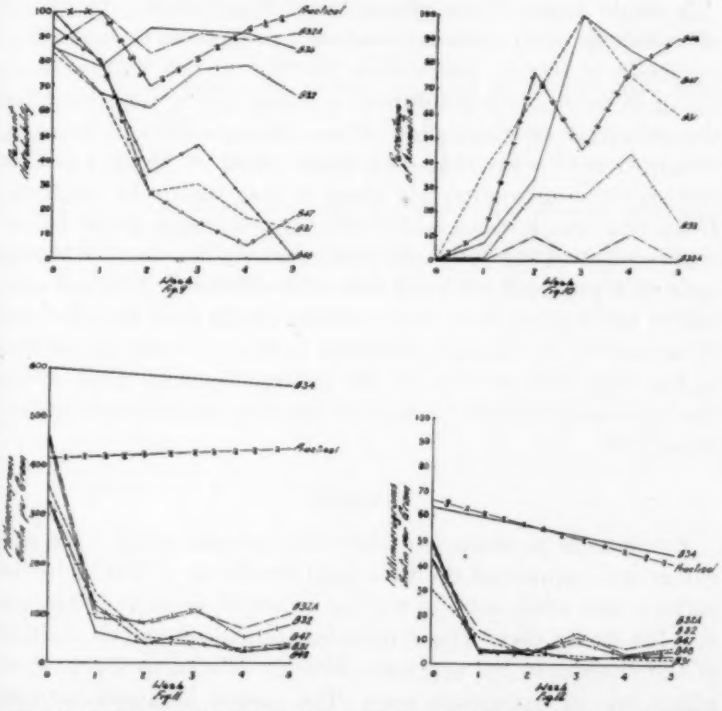

Fig. 9 Effect on hatchability of fat additions to a low biotin diet: diet B31, sucrose; diet B32, dextrin; diet B32A, dextrin with 20% fat; diet B34, 0.2 mg biotin per kilogram; diet B47, 10% oat hulls; diet B48, 10% oat hulls with 9 parts fat.

Fig. 10 Effect of fat additions to a low biotin diet on the incidence of congenital deformities.

Fig. 11 Effect of fat additions to a low biotin diet on the biotin content of egg yolk.

Fig. 12 Effect of fat additions to a low biotin diet on the biotin content of egg white.

the mature fowl to a greater extent than did oat groats. It is possible that the hulls fed with the oats provided a more favorable medium for the growth of intestinal microorganisms than that provided by oat groats. If this is the case, the addition of a fibrous product to a dextrin diet such as B32 might further enchance the synthesis of biotin.

It would be of considerable interest to determine the effects of feedstuffs other than oats and oat products on the intestinal synthesis of biotin. Some data bearing on this subject were obtained by running essentially a balance trial with hens fed the practical all-mash diet. From these results it was determined that a hen ingested about 10 μg of biotin per day and excreted approximately 20 μg of the vitamin in the feces. If an egg was laid an additional 6 μg of biotin could be accounted for. Thus it is apparent that in the case of laying hens on a practical all-mash diet, approximately two and one-half times the quantity of the vitamin in the food ingested can be accounted for through excretion in the feces and deposition in the egg. The portion of the gastrointestinal tract where this synthesis of biotin occurs is a problem under investigation at present.

SUMMARY

Evidence is presented to show that ground whole oats and oat groats supported the intestinal synthesis of biotin in the mature fowl when used to replace either 20 or 40% of sucrose in a low biotin diet. The synthesis, absorption and deposition of the vitamin in the egg were directly related to the level of whole oats or oat groats used. The results obtained indicate that whole oats stimulated the synthesis of the vitamin to a greater extent than did the oat groats. Oat hulls did not favor the intestinal synthesis of biotin under the conditions of this experiment.

An indication was obtained to the effect that 20% fat with dextrin favored the intestinal synthesis of biotin to a greater extent than 5% fat with dextrin.

ACKNOWLEDGMENTS

We are indebted to Wilson Laboratories, Chicago, Illinois, for the liver fraction L, and to Merck and Company, Rahway, New Jersey, for the biotin.

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THE IMPORTANCE OF BULK IN THE NUTRITION OF THE GUINEA PIG[1]

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(Received for publication September 7, 1948)

In spite of the rather extensive studies on the nutritional requirements of the guinea pig (cavy) it still is not possible to devise a purified ration that will produce "normal" rates of growth. Cannon et al. ('45) reported that growth of less than 7 to 8 gm per day from the second to the 8th weeks of life cannot be considered "normal" if growth on natural foodstuffs is accepted as the standard. These same workers concluded that growth and survival on purified rations, supplemented with linseed oil meal and solubilized liver, was poorer than on a crude diet and that the animals fed the former developed anemia and leucopenia. Woolley and Sprince ('45) described three guinea pig factors (abbreviated, G.P.F.) numbered 1, 2, and 3. They stated that G.P.F.-1 was folic acid, G.P.F.-2 was replaceable by a mixture of cellulose and protein, and G.P.F.-3 was an as yet unidentified factor present in solubilized liver extracts. However, the best growth obtained when all three of these factors were added to a purified basal ration was only 4 gm per day during a period of 4 weeks.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; also by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association.

The guinea pig, unlike the rat, is an herbivorous animal, subsisting entirely on foods of plant origin. Therefore, it is not surprising to find that the digestive tract is different, in that it includes a large functioning caecum constituting about 15% of the body weight. The results of the present investigation will be cited in an attempt to relate this peculiarity in the lower intestinal tract to the importance of bulk in a purified ration.

EXPERIMENTAL

There is abundant evidence to indicate that the guinea pig is very susceptible to certain diseases, especially *Salmonella* infections and pneumonia. For this reason two precautions were taken: first, the animals were obtained from a single source during the entire investigation and, secondly, the animals were housed in a room of uniform temperature (75 to 85°F.), in individual wire bottom cages which were steam sterilized before being used. Water was supplied by means of bottles hung on the outside of each cage with a glass tube and drinking tip on the inside of the cage.

A commercial ration [2] was used as the positive control diet. It is known to be high in alfalfa leaf meal and has always resulted in excellent growth when used for our animals. The basal synthetic diet had the following composition: Sucrose, 60; casein [3] (vitamin free), 30; salts IV, 4; fortified soybean oil, 4; sucrose mixture containing B vitamins, 0.8; and choline, 0.3 parts. The fortified soybean oil was prepared by dissolving β-carotene in chloroform and distributing it as a layer on the sides of a round-bottom flask while removing all of the chloroform with reduced pressure. Soybean oil was added to the flask and heated (60–70°) on a sand bath for three or 4 hours until the carotene was dissolved in the oil. The solution was allowed to cool and crystalline vitamin D_2 and vitamin K as menadione were added. Finally alpha-tocopherol was added and the fortified oil made up to a final weight. The use

[2] Rockland guinea pig pellets.

[3] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

of 4% of this oil supplied 1.2 mg of β-carotene, 12 mg of α-tocopherol, 8 μg of calciferol, and 0.2 mg of vitamin K per 100 gm of ration. The added mixture of B vitamins supplied 1 mg of thiamine hydrochloride, 1.4 mg of riboflavin, 1 mg pyridoxine, 3 mg calcium pantothenate, 200 mg inositol, 10 mg niacin, 10 mg *p*-aminobenzoic acid, 0.04 mg biotin, and 0.3 mg folic acid per 100 gm of ration. Vitamin C was supplied by dissolving sufficient ascorbic acid in a 70% sugar solution to secure 100 mg per milliliter. This solution was fed with individual pipettes at a level of 0.25 ml every other day, which was equivalent to 12.5 mg per animal per day.

Loss of guinea pigs during the first few weeks on experiment was completely eliminated in most groups of animals if small amounts of the stock ration were placed on top of the experimental diet during the first 7 days. Approximately 4 or 5 gm were used the first day and the amount decreased each day until no addition was made after the 7th day. In spite of these precautions, a high mortality was observed in a few of the experimental groups. No explanation could be found for these results, and when the condition was encountered the entire group of animals was discarded. It should be emphasized that animals from these groups never failed when fed the stock ration.

RESULTS

The growth data are given in table 1. Since the rate of growth of the animals in different groups varied to some extent, the results are separated into several series. When the commercial stock ration, supplemented with vitamin C, was fed, an average daily gain in weight of 6.9 gm was obtained over a 6-week period. The basal synthetic ration, on the other hand, which contained all of the known dietary factors except vitamin B_{12}, gave a growth response of only 1.8 gm per day. When crude casein was used in place of the vitamin-free casein the same results were obtained. No improvement resulted when dextrin was used in place of sucrose. Since one of the major components of the stock ration was alfalfa leaf

TABLE 1

Growth of guinea pigs on different rations[1]

	NO. OF ANIMALS	WEEKS ON EXP.	AVERAGE GAIN IN WT.
			gm/day
Series I			
Rockland stock diet	30	6	6.9
Basal synthetic	9	6	1.8
Basal synthetic (crude casein)	7	6	1.9
Basal synthetic (dextrin)	3	6	1.9
Basal synthetic + 15% alfalfa leaf meal	4	6	3.6
Basal synthetic + 25% alfalfa leaf meal	4	8	6.5
Basal synthetic + 25% autoclaved alfalfa leaf meal	5	6	6.5
Basal synthetic + 30% beet pulp	4	6	6.8
Basal synthetic + 10% beet pulp fiber	4	6	2.0
Basal synthetic + 20% beet pulp fiber	2	6	3.1
Basal synthetic + 20% pectin	3	6	4.4
Basal synthetic + 20% gum arabic	3	6	5.2
Basal synthetic + 15% cellu flour	9	6	4.6
Series II			
Basal synthetic + 15% gum arabic	20	6	5.1
Basal synthetic + 15% xylose	3	3	3.5
Basal synthetic + 2% levulenic acid	4	4	2.5
Basal synthetic + hydrolyzed gum arabic ≅ 15%	5	4	2.5
Series III			
Basal synthetic + 15% gum arabic + summer milk ad lib.	3	6	4.7
Basal synthetic + 15% gum arabic + extract of beef liver ≅ 10%	5	6	5.7
Basal synthetic + 15% gum arabic + 15% brewers' yeast	5	6	6.7
Basal synthetic + 15% gum arabic + 4% fish solubles	6	6	2.5
Basal synthetic + 15% gum arabic + 6% grass juice powder	6	6	4.6
Basal synthetic + 15% gum arabic + 4% grass juice	6	6	2.8

TABLE 1 *(continued)*

	NO. OF ANIMALS	WEEKS ON EXP.	AVERAGE GAIN IN WT.
			gm./day
Series IV			
Rockland stock diet	4	9	6.6
Basal synthetic + 15% gum arabic	5	9	5.1
Basal synthetic + 15% gum arabic + alfalfa ash ≅ 25%	5	9	5.4
Basal synthetic (crude casein) + 15% gum arabic	5	9	5.5
Basal synthetic (crude casein) + 15% gum arabic + alfalfa ash ≅ 25%	6	9	7.0
Basal synthetic (crude casein) + 15% gum arabic + stock ration ash ≅ 100%	4	9	5.7
Basal synthetic + alfalfa ash ≅ 25%	5	9	4.4
Series V			
Basal synthetic + 15% gum arabic	10	6	5.1
Basal synthetic (20% casein) + 15% gum arabic	10	6	2.3
Basal synthetic (20% crude casein) + 15% gum arabic	9	3	2.4
Basal synthetic (20% casein) + 15% gum arabic + 0.3% L-cystine + 0.1 DL-tryptophan	5	6	4.1
Basal synthetic (20% casein) + 15% gum arabic + 1% DL-arginine + 0.1% L-cystine + 0.2% glycine	2	6	2.5
Basal synthetic (15% casein) + 15% gum arabic + 15% wheat gluten	3	6	4.7
Basal synthetic (8% casein) + 15% gum arabic + 8% fibrin + 8% egg albumin	3	6	4.2
Basal synthetic (20% casein) + 15% gum arabic + 7% protolysate	4	3	2.7
Basal synthetic (20% casein) + 15% gum arabic + extract of crude casein ≅ 50%	6	3	3.0
Basal synthetic (30% special casein) + 15% gum arabic	5	4	5.1
Basal synthetic (18% casein) + 30% beet pulp	4	6	5.1
Basal synthetic (28% casein) + 30% beet pulp	4	6	5.2

[1] All additions made at the expense of the sucrose in the basal ration.

meal, experiments were set up to determine if the addition of this material would improve the growth of the animals. When 25% of leaf meal was added to the basal synthetic ration the average rate of growth was equal to that obtained on the stock ration, but 15% of the meal was definitely inadequate. Apparently alfalfa supplied all of the missing dietary factors when added at the higher level. The effect of the alfalfa was not altered when the meal was autoclaved.

Woolley and Sprince ('45) recognized dietary bulk as an important factor in the growth of guinea pigs and used cellu flour and cellophane to satisfy this requirement. In order to study the effect of bulk more extensively, it was decided to try beet pulp, a material which contains more than 20% crude fiber. Plain dried beet pulp was added to the ration at a level of 30%. When this addition was made the gain in weight over a 6-week period was equal to or better than that obtained with 25% alfalfa leaf meal. However, when the crude fiber from beet pulp was isolated and fed at levels of 10 and 20%, poor growth resulted. These results were somewhat unexpected and led to an examination of the chemical analysis of beet pulp. It was found that beet pulp contains as much as 30% of pectin, which is a hemicellulose (Codling and Woodman, '29).

The following materials were therefore tested in order to differentiate between the effects of cellulose and hemicellulose: Cellu flour, cellophane, pectin,[4] gum arabic, gum mesquite, gum tragacanth, oat straw, agar, cornstarch, potato starch, paper pulp, wood shavings, silica gel and methyl cellulose. Of all of these materials, powdered gum arabic consistently produced the best response. Pectin, agar, oat straw, cellu flour and cellophane stimulated growth to some extent but to a lesser degree than gum arabic. The other materials were either inactive or only slightly active.

Many of the animals receiving the basal ration plus gum arabic were continued on the ration after the 6-week period,

[4] Obtained through the courtesy of the California Fruit Growers Exchange, Ontario, California.

and these animals continued to grow and showed no indication of a serious deficiency during a period of 8 months or more. Post-mortem examination of guinea pigs failing to survive on the basal ration without gum arabic revealed no specific abnormalities. One symptom which was noted was that the animals ate the hair on the abdomen and sides of the body. This was observed in animals on almost all the inadequate rations but never in more than two out of 5 cavies on the same ration. Another symptom which was frequently observed on adequate as well as inadequate diets was diarrhea. The duration and severity of the condition varied greatly. Occasionally rapidly growing animals would suddenly lose as much as 50 gm in body weight, at which time a severe diarrhea developed. However, all of the animals on any given ration did not exhibit such tendencies. The feces from animals receiving the stock rations were always hard and dry in appearance, whereas the feces from guinea pigs on purified rations were unusually soft, even when the rate of growth was normal.

When it was found that gum arabic was active in promoting growth there were at least two questions to be answered; first, how does the gum function and, secondly, are there any other missing dietary factors to be contended with when a ration containing 15% gum arabic is fed? Gum arabic contains 28.3% galactosoglucuronic acid, 29.5% galactose, 34.4% arabinose and 14.2% rhamnose hydrate. Arabinose was not available, but in order to test the activity of a pentose sugar, xylose [5] was fed at a level of 15%. Poor growth resulted (series II). Davis and Briggs ('47) reported that materials like Ruffex, wood shavings, and cellu flour were capable of stimulating the growth rate of chicks when added to a purified ration at levels of 5 to 15%. These investigators also found that the degradation products of cellulose, furfural and levulenic acid, were equally active. Guinea pigs were fed a ration containing 2% levulenic acid but negative results were obtained. The most critical experiment designed to determine

[5] Obtained through the courtesy of the Northern Regional Research Laboratory, Peoria, Illinois.

whether the gum could be replaced by degradation products involved hydrolysis of gum arabic with dilute H_2SO_4 (Butler and Cretcher, '29), removal of the sulfate with $CaCO_3$, and then addition of the filtrate to the purified ration. Again the results, as judged by the average gain in weight, were subnormal and it was concluded that the intact gum molecule was essential for growth-promoting activity, probably due to a "bulk" effect on the lower intestinal tract.

The average growth rate for 20 animals receiving a ration containing 15% gum arabic was 5.1 gm per day (series II). Since a growth rate of 6.9 gm per day was obtained on the stock ration, it appeared that gum arabic was not the only factor involved. Various natural supplements were added to a ration containing 15% gum arabic, including raw summer milk (ad libitum) fish solubles, grass juice powder,[6] grass juice (20% solids),[7] a liver extract and yeast (series III). Yeast produced a good response and the methanol extract of beef liver produced some response but the other supplements, namely milk, fish solubles, grass juice powder, and grass juice, appeared to depress rather than enchance growth.

Since 25% alfalfa leaf meal was capable of bringing about a normal growth rate, fractionation of this material was undertaken. It was found that an ether extract, a 90% ethanol extract, a methanol extract, or hot water extracts were all less active than the residues from which these extracts were prepared. The next logical fraction to test was the ash. Surprisingly enough, the ash of alfalfa leaf meal showed some effect when added to purified rations either with or without gum arabic (series IV). In fact, nearly normal growth rates were obtained when a ration containing 15% gum arabic plus alfalfa ash equivalent to 25% was fed. This ration has been tested several times and the results were nearly always in the normal range during the third to the 6th week of the experiment. The growth rate was often subnormal for the first three

[6] Obtained through the courtesy of the Cerophyl Laboratories, Kansas City, Missouri.

[7] See footnote 5.

weeks of an experiment, after which nearly normal growth occurred. Whether this initial failure in growth is due to a missing dietary essential or simply a refusal on the part of the guinea pig to accept this ration readily is not known.

Stock ration ash as the only source of minerals in the ration (the ash from 100 gm of stock ration was substituted for 4 gm of salts IV) was as effective as alfalfa ash. The use of crude casein had some beneficial effect, especially in the presence of alfalfa ash. However, when the animals were continued on this ration for 12 weeks there was little difference between the animals receiving the purified and the crude casein.

Attempts have been made to determine what particular constituent in the alfalfa accounts for the activity observed. Cobalt, boron and molybdenum have been added singly and together without success. Sodium, magnesium, calcium and phosphorus have also been eliminated as being involved. The level of salt mixture in the basal ration (salts IV, Hegsted et al., '41) was raised from 4 to 8% with no improvement. The ash of alfalfa is quite basic and this may exert a favorable effect on some of the microflora of the lower tract by raising the pH. Another possibility which must be explored is the sodium-potassium ratio, because alfalfa ash contains a high level of potassium. Further work is in progress to clarify this question.

Van Wagtendonk and Wulzen ('43) observed the development of a wrist-stiffness in guinea pigs on a ration containing vitamin-fortified skim milk powder plus wheat straw. The deficiency could be corrected by feeding fresh cream or cane juice or crystalline material isolated from these sources. This wrist-stiffness has never been observed in the investigations reported here, and there was no response as measured by growth when either ergostanol [8] (Oleson et al., '47) or the Wulzen anti-wrist stiffness factor [9] was added to a purified ration. The

[8] Obtained through the courtesy of the Lederle Laboratories, Pearl River, New York.

[9] Obtained through the courtesy of the Lilly Research Laboratories, Indianapolis, Indiana.

possibility also arose that vitamin P active compounds such as hesperidin might be stimulatory for growth, but here again negative results were obtained when this substance was tried.

One other question was investigated, namely, the necessity for a high level of casein (30%) in the basal ration. If the level of casein was reduced from 30 to 20% in a ration containing 15% gum arabic, the growth was markedly reduced (series V). It will be recalled that Woolley and Sprince ('45) recognized this fact and they were able to secure growth equal to that obtained with 30% casein by adding arginine (1.0%), cystine (0.1%), and glycine (0.2%) to a ration containing 20% casein. The addition of several amino acids to the 20% casein ration did not improve growth (series V). The commercial stock ration contains only 18% crude protein and excellent growth is always obtained when it is fed. Why then should purified rations containing 20% casein fail? Kuiken et al. ('44) concluded that crude casein contained an unknown dietary factor essential for guinea pigs. Several other experiments were conducted including the addition to purified rations containing gum arabic of wheat gluten, a pancreatic digest of casein,[10] fibrin, egg albumin, and laboratory-prepared cold acetic acid precipitated casein. None of these supplements was able to increase the growth rate to that obtained with 30% casein. Quite recently two rations were compared, both containing 30% beet pulp but one with 18 and the other with 28% crude casein. It was rather surprising to find that the growth rates at the end of 6 weeks were practically the same (5.1 and 5.2 gm per day). These rations also contained dextrin in place of sucrose and cellu flour (3%). However, the fact that the ration containing 18% crude casein produced as good response as the one containing 28% suggests two possibilities: Either the protein (2%) supplied by the 30% beet pulp was an efficient supplement to the casein, or the lower level of protein is adequate when a proper source of bulk is present.

[10] "Protolysate."

DISCUSSION

The results presented in this paper clearly demonstrate the beneficial effect of gum arabic when added to a purified diet for guinea pigs. The exact mechanism by which this gum functions is still unknown. It is known that the class of gums to which this one belongs are hydrophylic in nature and hence have considerable power to retain water and to give colloidal suspensions. Such a gum may function by affecting the type of bacteria multiplying in the lower part of the digestive tract. The gum may favor certain organisms which are able to synthesize unknown factors which are then made available to the host either by direct absorption, by coprophagy or by both mechanisms.

On the other hand, the hemicellulose may tend to decrease the number of unfavorable organisms. Diarrhea which might well owe its origin to an abnormal intestinal microflora, was frequently noted when guinea pigs were fed the unsupplemented rations. Furthermore, the addition of ash from alfalfa has a definite effect and this effect is more apparent in the presence of gum. Further studies are needed to determine which of the mineral elements may be concerned. Perhaps the most important factor is the proper balance among the ash elements that are present in predominant amounts. It is also interesting that the presence of hemicellulose material, especially in the form of beet pulp, tends to decrease the protein requirement. There is little reason to believe that the casein itself supplies a limiting factor; instead, the necessity for the higher level is probably related to the lack of bulk in the diet.

SUMMARY

A purified basal ration containing all of the known nutrients (except vitamin B_{12}) produced a rate of growth of only 1.8 gm per day for 6 weeks compared to a normal rate of 6.9 gm per day when a commercial ration composed of natural feeds was fed. When the purified basal ration was supplemented with 25% alfalfa leaf meal (in place of an equal amount of

sucrose) a nearly normal average growth rate of 6.5 gm per day was obtained. When the basal ration was supplemented with 30% dried beet pulp growth was equally good. Powdered gum arabic (a hemicellulose) was consistently the most active single supplement when fed at a level of 15%. However, the average growth rate of 5.1 gm per day when this gum was fed was less than the normal rate of 6.9 gm. When the ash from alfalfa leaf meal equivalent to 25% was added to a ration containing 15% gum arabic, nearly normal growth rates were obtained for periods as long as 12 weeks. The level of protein in the synthetic ration could be reduced when gum arabic was fed.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

IV. INFLUENCE OF ACTIVITY [1]

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(Received for publication September 22, 1948)

In recently published work from this laboratory Forbes et al. ('46a, '46c), working with growing albino rats, have shown that dietary fat confers economy of food utilization. In their experiments, diets varying in fat content from 2 to 30% were fed so as to supply equal quantities of energy and of protein and a considerable surplus of all known vitamins. Statistically significant results were obtained for gains in nitrogen and energy and decrease in heat production, in the order of increasing fat content of the diets.

In experiments with mature rats, using the same diets fed in isocaloric quantities and supplying the same protein and vitamin intakes (Forbes et al., '46b, '46d), the heat increment or energy expense of utilization of the diet was similarly found to decrease as the fat content of the diet increased.

Each of these investigations consisted of a series of two separate trials, one with greatly increased intakes of 10 of the vitamins. This was done to eliminate the possibility that results were influenced by a suboptimum intake of one or more of the vitamins. However, no significant changes were observed due to the higher level of vitamin intake.

[1] Authorized for publication on September 18, 1948 as paper No. 1473 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

During the course of this work it became evident that the amount of energy expended in heat production, other than the dynamic effect, varied with the intake of fat and was of importance in explaining the differences in total heat production, particularly among the growing rats.

While this work had not shown any consistent difference in metabolizable energy on the various levels of fat intake, it had shown a marked difference in heat increments where growth was not a factor. Therefore, the question of the heat production which resulted from activity was of interest, and especially its possible relation to the intake of different amounts of fat.

No report dealing directly with this particular subject is known to the present authors. Of interest, however, is the work of Scheer, Dorst, Codie and Soule ('47), who found that the physical capacity of rats increased as the fat content of the diet increased from 0 to 40%. Total activity for the entire day was not measured, but the rats on high fat diets were capable of a more sustained period of excercise than those on low fat rations.

The evaluation of activity in terms of calories presents a very difficult problem. An indirect approach to it was made by measuring the total heat production under conditions of normal cage activity and again with activity restricted to a minimum, all other imposed conditions being the same. The difference was considered to represent the energy expenditure associated with activity. Obviously, heat increment includes energy derived from the "work of digestion" of the food, which is a form of activity but, as such, is of interest in this connection only as it is related to dynamic effect.

The work here reported was done in two parts, the first being a measure of the total heat production, with activity included. in mature male rats fed two distinctly different levels of fat intake, 2% and 30% of the diet. In the previous work (Forbes et al., '46a, '46c) in which the total heat production was determined, growing rats were used; it was de-

sired in the present experiment to eliminate the variable of growth by employing physiologically mature animals.

The second experiment was the direct result of the first and was planned to measure the heat production with and without activity on alternate days, the reason for this being that previous conclusions drawn from work done at different times and on different animals would be more clearly established if completed on the same animals with a minimum of time elapsing between the two determinations.

EXPERIMENT NO. 1

Total heat production, with activity included, of mature rats at different levels of fat intake

In this experiment two groups of 12 male rats each were selected on the basis of uniformity of weight and age and placed in individual cylindrical cages 8 inches in diameter. The animals were approximately 6 months of age at the start and weighed an average of 390 gm. During a preliminary period of one week a constant feed intake of the colony diet was fed at about the same level of energy intake as was planned for the experimental ration to follow. One group of 12 rats was then placed on the low fat diet and the other group was given the high fat diet. The feed intake was maintained constant from this point for the remainder of the experiment, with each animal receiving the same amount of energy and protein each day. After one week of preliminary feeding a 10-day collection of feces and urine was made in metabolism cages which have been used extensively in this laboratory for the separate collection of feces and urine (Swift, Kahlenberg, Voris and Forbes, '34). The one variation made from the method as originally described was that at the beginning of the collection period 100 ml of a preservative solution containing 2 gm of cupric sulfate and 1 gm of sodium fluoride were evaporated to dryness in the crystallizing dish in which the urine was collected. This dish with the preservative was washed out only at the end of the 10-day collection period and not each day.

Following the collection period, the CO_2 production was measured continuously for two days, using respiration cages sufficiently large to accomodate an individual rat cage (Forbes, Swift and Black, '38). The animals therefore had the same freedom of movement during the time when the heat production was being measured as they normally had.

A second period for collection of excreta immediately followed the first test in the respiration chambers, and this was followed by another CO_2 measurement. The diets were then reversed and after the usual preliminary period to establish the animals on the new feed a series of two more excreta collections and CO_2 production periods was instituted. In other words, each rat, with a few exceptions due to refusal of feed, had two periods on the low fat and two on the high fat diet, both for collection of excreta and for determination of the carbon dioxide. The actual number of rats completing each period is shown in table 1. Heat production in all cases was computed by the carbon-nitrogen balance method as described by Forbes, Swift and Black ('38) and as indicated in table 2.

A correction for a CO_2 blank, not considered in the original procedure, was made in this experiment as it was found that a small amount of CO_2 was always obtained in blank runs when excreta were present in the collection dishes in the respiration cages. During the respiration measurements excreta were collected under a layer of lightweight paraffin oil and 100 ml of the copper sulfate-sodium fluoride preservative solution. Although this solution completely covered both the urine and feces as they fell into the dish after being voided, there was apparently some decomposition taking place before the end of the two-day run, with resultant production of CO_2. The amount of CO_2 actually obtained from an average of 18 blank runs with one-day collection of excreta in the respiration chamber was 26 mg.

The diets fed were prepared to be as nearly identical as possible to those used in earlier studies of the relationship between fat and economy of food utilization (Forbes, Swift, James, Bratzler and Black, '46c). The high level of vitamin

intake referred to was provided in all the diets in this and the following paper, in order to avoid any possible influence resulting from the suboptimum intake of an essential nutrient. The feed intake was maintained constant for the entire experiment for all rats on each diet, thus supplying the same energy and protein and essentially the same amount of vitamins and minerals per rat per day.

TABLE 1

Experiment 1. Partition of average daily nitrogen intake per rat

NITROGEN DATA	PERIOD 1		PERIOD 2		PERIOD 3		PERIOD 4	
	Fat in diet		Fat in diet		Fat in diet		Fat in diet	
	2%	30%	2%	30%	2%	30%	2%	30%
	12 animals		11 animals		9 animals		9 animals	
Intake mg	542	549	542	549	542	549	542	549
Digested mg	491	503	491	504	494	506	493	503
% of intake	90.6	91.6	90.6	91.8	91.2	92.2	91.0	91.6
Urine mg	469	479	449	455	433	451	448	459
% of intake	86.5	87.2	82.8	82.9	79.9	82.1	82.7	83.6
Retention mg	22	24	42	49	61	55	45	44
% of intake	4.1	4.4	7.7	8.9	11.3	10.0	8.3	8.0

Nitrogen was determined by the standard Kjeldahl procedure and energy by the Emerson bomb calorimeter. Carbon was determined by a wet combustion method similar to that described by Furman ('39), in which the carbon is oxidized to CO_2 by a mixture of sulfuric, phosphoric and chromic acids. The CO_2 evolved was then collected in soda lime contained in glass stoppered U-tubes and weighed.

DISCUSSION OF RESULTS (EXPERIMENT NO. 1)

In table 1 the partition of nitrogen for the 4 periods is presented, showing the intake, excretion and body gain. The average live weights of the rats for periods 1, 2, 3, and 4 were, respectively, 364, 377, 400 and 403 gm for the 2% fat diet, and 362, 383, 392 and 397 gm for the 30% fat diet.

In each of the 4 periods the rats receiving the 2% fat diet excreted more nitrogen in the feces and less in the urine than

TABLE 2

Experiment 1. Intake and partition of daily food energy

FOOD ENERGY DATA	PERIOD 1		PERIOD 2		PERIOD 3		PERIOD 4	
	Fat in diet		Fat in diet		Fat in diet		Fat in diet	
	2%	30%	2%	30%	2%	30%	2%	30%
	Cal.	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
Intake	61.52	61.52	61.52	61.52	61.52	61.52	61.52	61.52
Feces	2.95	2.75	2.90	2.80	2.87	2.85	2.98	2.77
Urine	4.03	4.12	3.87	3.92	3.73	3.88	3.85	3.94
Metabolizable	54.54	54.65	54.75	54.80	54.92	54.79	54.69	54.81
Body gain								
As protein	0.77	0.81	1.40	1.66	2.10	1.90	1.56	1.52
As fat	7.94	8.55	6.45	5.92	5.48	6.20	5.53	5.81
Total body gain	8.71	9.36	7.85	7.58	7.58	8.10	7.09	7.33
Heat production[1]	45.83	45.29	46.87	47.23	47.34	46.69	47.60	47.47
Coef. of variation (heat production)	3.6	4.8	4.2	3.5	5.9	3.3	5.5	3.5

[1] Includes normal activity.

did the rats consuming the high fat diet. These differences are insignificant, and the total nitrogen excretion in the feces and urine was essentially the same on both diets.

Table 2 gives a summary of the average daily energy balances for the 4 periods on the high and low fat diets. The high metabolizable energy of both essentially synthetic diets was due to the lack of any bulk or roughage. Slight differences are noted between the energy losses in the feces and urine, but since these are in opposite directions the metabolizable energy values are closely similar.

To determine heat production by the carbon-nitrogen balance method the experimental animals must be thoroughly established on a constant feed intake before any heat measurements can be made that will be thoroughly representative of the conditions imposed. The level of feed intake chosen must not, therefore, be greater than the animals will consume regularly and is always less than on ad libitum feeding. When the rats were first removed from the colony diet and given a weighed amount of the experimental ration, a decrease in weight occurred for several days, some of which was doubtless "fill." After adjustment to a constant amount of the new feed there was a small gain of energy, ranging from 7 to 9 cal. per day throughout the experiment.

In estimating the amount of feed to be given daily at the beginning of the experiment it was known that the theoretical objective of no change in body weight was impossible, but an attempt was made to have the balance on the positive side instead of the negative as losses in body tissue involve a greater loss of heat (dynamic action) than do gains in tissue.

The daily heat productions were similar for both diets in each of the 4 periods, although the trend in three was for the rats on the low fat diet to have a slightly higher heat production than those on the high fat diet.

This uniformity in heat production was somewhat surprising, since the heat production as measured by the body balance procedure in growing rats was greater on the low fat diet than on the high fat diet, with equal energy intakes (Forbes, Swift, James, Bratzler and Black, '46c). The one big difference between these two experiments was in the age of the rats and, consequently, in their physiological status. In the young, rapidly growing animal growth is undoubtedly one of the first, if not the first, demand made upon the energy available to the animal after the needs of vital activities have been satisfied. When the animal has reached maturity, as was the case in the present experiment, the urge to grow has practically ceased and the intake as well as the expenditure of energy was governed by factors other than growth.

In view of the results of this experiment, which show essentially the same heat production as well as metabolizable energy on a high and a low fat intake, and in view of the earlier work in which it was found that the dynamic effect was less on the high fat diet, at least under the experimental conditions imposed, it may be concluded that on high fat diets more heat remains to be dissipated in various forms of activity. It is recognized that this conclusion is drawn from data in two separate experiments conducted at different times and on different animals, but the evidence, as obtained, points to a distinctly greater amount of activity in the rats on the high fat diet.

EXPERIMENT NO. 2

Heat production of mature rats, with and without activity, at different levels of fat intake

The second part of the work reported in this paper originated with the idea that it would further strengthen the data obtained to plan an experiment in which the heat production was measured on consecutive days in the same animals with activity restricted in one case and with normal cage activity in the other. The conclusion drawn above that activity is greater in rats receiving high fat diets than in those on low fat diets is dependent, in part, on results from a study of heat increments (Forbes et al., '46d), and for this reason might possibly be criticized in that it is based on work completed under different conditions and at different times. The technique of combining the entire procedure into one experiment would not only satisfy this criticism but also give additional information on the question of total heat production with unrestricted activity at different levels of fat intake.

Two groups of mature male rats were selected, 10 animals in a group, to be established on rations containing 2 and 30% of fat. The rations were identical with those used earlier and contained the high level of vitamin intake. The rats were also selected from the same group of animals used before but were not all the identical animals. Their age had increased by 6

months and at the time respiration measurements were started they were approximately one year old.

The experimental routine for collection of excreta and care of the animals was essentially the same as that reported in the first part of this paper. The excreta were collected for one period of 9 days' duration in this case and the collection always preceded the respiration measurements.

The total heat production, with activity unrestricted, was measured by the carbon-nitrogen balance method for two complete days, as explained earlier. The heat production with activity restricted was measured for 7 hours by the open-circuit respiratory quotient Haldane procedure with equipment used routinely in this laboratory. In this procedure the rats were confined in half-gallon Mason type jars for respiration chambers and a bright light was placed immediately overhead to keep them quiet. This has proved to be the most satisfactory method of restricting activity which the writers have tried. To eliminate figures for periods when activity was unavoidably high, the CO_2 production was determined hourly and those hours which differed from the average of the final 6 hours by more than $\pm$ 6% were excluded.

An effort was made to standardize conditions in every way possible in order to maintain uniformity of metabolism. These precautions included maintenance of a constant room temperature by thermostatic control at a point slightly above the critical temperature for the rat, and exact regularity in time of feeding. The animals were familiarized with the apparatus by being placed in it for several hours during the preliminary feeding period, the respiration measurements being so arranged that for about half the animals the Haldane method preceded by one day the carbon-nitrogen balance method, while for the others the order was reversed and the Haldane method was used last instead of first.

DISCUSSION OF RESULTS (EXPERIMENT NO. 2)

In table 3 are presented the average daliy intake and balance of nitrogen for the rats on the two diets. The high

digestibility of the nitrogen is apparent again in this experiment, as is the close similarity of the nitrogen in the urine on the two diets. The retention of nitrogen reflects the aim of the feeding schedule, in which an effort was made to approximate the maintenance level of intake yet to have a small positive balance. Retention figures are given mainly to show the status of the animal relative to the plane of nutrition and not so much to demonstrate the efficiency of utilization of this component of the diet. To do this, planes of nutrition other than maintenance would be included.

The significance of the nitrogen utilization figures lies in the close agreement of results between the two diets, indicating that the fat intake did not influence retention of nitrogen.

TABLE 3

Experiment 2. Partition of average daily nitrogen intake per rat

FAT IN DIET	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION
%	*mg*	*mg*	*% of intake*	*mg*	*% of intake*	*% of intake*
2	505	464	91.9	456	90.3	1.6
30	504	468	92.9	464	92.1	0.8

In one previous instance (Forbes et al., '46a) nitrogen retention was in the order of increasing fat content of the diet, but in all subsequent work no difference has been found.

The metabolizable energy values of the daily food, as reported in table 4, show that a somewhat greater amount of metabolizable energy was furnished by the low fat diet. Due to the consistency of these figures the odds that this difference is significant are extremely high, even though the actual difference is small. In previous work using these rations, and in the latest publication in this series of experiments where low protein rations of different fat content were fed (French, Black and Swift, '48), the metabolizable energy has been relatively similar irrespective of the fat content of the diet. The greatest difference observed in any of these experiments

was 2% (Forbes et al., '46c), this difference being in favor of the low fat ration and therefore comparable to the present finding. The significance of these observations must necessarily be considered from its broader aspect and not from the point of view of a single observation. In this respect the fat content of the rations did not consistently affect its metabolizable energy, but when a difference was noted it was in a decreasing order as the fat content of the diet increased. This point emphasizes the need for caution in interpreting small though apparently significant differences in biological work.

Heat production in this experiment as measured with activity either restricted or unrestricted follows closely the pattern of the previous experiments and thereby confirms the results

TABLE 4

Experiment 2. Intake and partition of daily food energy

FAT IN DIET	INTAKE	FECES	URINE	METABOLIZABLE ENERGY	BODY GAIN	HEAT PRODUCTION			
						With activity included	Coef. of variation	With activity excluded	Coef. of variation
%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	%	*Cal.*	%
2	57.67	2.46	3.98	51.23	7.72	43.57	4.0	38.44	6.9
30	57.67	3.66	4.02	49.99	7.97	42.06	2.9	36.15	7.1

obtained, especially as reported in the first part of this paper. This is an important point and indicates that a number of variable factors such as different animals, time of year, experimental routine, et cetera, did not materially influence the results obtained. Through use of the technique of having the respiration measurements made with the same animals on consecutive days there can be little doubt of the part that activity plays in total heat production. It is recognized that one reasonable possibility remains which could influence the respiration measurements obtained with the Haldane method, since the period of observation does not extend over a full day and any change in metabolism during a time other than when the measurements were being made would not be re-

flected in the final result. No claim ignoring this possibility has been made in any of the previous papers and, in fact, it has been specifically stated that the conclusions drawn applied only to the manner of experimentation employed.

With this general idea in mind, some preliminary work was initiated in this experiment which indicates the possible existence of well-defined changes in the curve of total heat production over a 24-hour period. These fluctuations in daily heat production, both with and without activity, have been investigated in detail and will be reported in a later paper.

The observed difference in total heat production between the two groups of animals of 1.51 Cal. per day (table 4) is the largest yet obtained. Although the odds that this difference is significant are 38 to 1, little emphasis is placed on it since the work taken as a whole has shown no significant difference in heat production between the two groups of mature rats when determined by the carbon-nitrogen balance method with activity unrestricted.

Contrasted with the similarity in heat production observed when activity was uncontrolled is the greater difference when activity was limited. In this experiment it is noted that the difference in average daily heat production between the two diets was 2.29 Cal., or, expressed in another way, the rats on the low fat ration produced 6.3% more heat than those on the high fat ration. The odds that this difference is significant are 555 to 1. This finding confirms previous work when the Haldane procedure was used, the values reported in the two earlier papers showing that the rats on the low level of fat intake produced 10.7 and 8.7% more heat than those on the high fat ration at the maintenance level of intake (Forbes et al., '46b, '46d). This comparison can be made only on this level, since the supermaintenance levels, as used with the maintenance levels in the determination of heat increments, represent a much greater intake of food than was given in the course of the present work.

In conclusion it may be said that the total heat production with activity unlimited was not affected to any appreciable

extent by the level of fat intake, but the heat production as determined with activity restricted was markedly affected. This latter finding harmonizes with our previous observations, and since the metabolizable energy is essentially the same on either diet the remainder of the energy balance or energy expended on activity must therefore be greater on the high fat diet, varying reciprocally with the heat increment.

SUMMARY

In two separate experiments a study was made of the relation of dietary fat to the amount of spontaneous voluntary activity in the mature albino rat.

Due to the difficulty of measuring activity directly, it was estimated as the difference between the total heat production measured with and without activity.

In the first experiment the total heat production of two groups of 12 rats each was measured by the carbon-nitrogen balance method for 4 periods, each of two days' duration, with the animals being permitted normal freedom of movement during the respiration measurement. Equicaloric diets of either low (2%) or high (30%) fat content and containing the same amount of protein, vitamins and minerals were fed, with reversal of diets being made when the experiment was half completed.

It was found that the heat production, as well as the utilization of nitrogen, was not influenced appreciably by the level of fat in the diet. As the heat increment had previously been found to be greater on a low fat diet, it was concluded that the energy expended in all forms of activity must be greater in animals on a high fat diet, varying in a reciprocal manner with the heat increment.

To substantiate this claim relative to the amount of activity, a second experiment using the same animals was conducted in which the heat production was measured on consecutive days, first with activity unlimited and then with it limited. For the latter estimation the Haldane procedure was used, with the respiratory measurement lasting 7 hours.

The results obtained under both conditions of experimentation agree closely, in principle, with former observations showing approximately the same total heat production on a high fat diet when activity was permitted but a lower heat production when activity was limited.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

V. FLUCTUATIONS IN CURVE OF DAILY HEAT PRODUCTION [1]

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ONE FIGURE

(Received for publication September 22, 1948)

In the previous paper evidence was presented showing that the over-all activity of experimental animals increased as the fat content of the diet increased (Black, French and Swift, '49). This evidence also indicated that this increase in activity was related in a reciprocal manner to the heat increment. These findings were applicable to mature albino rats only, since it had been found in earlier work that the heat production of growing rats was less on a high fat diet than on a low fat diet (Forbes et al., '46a, '46c). This difference in results is attributed to the physiological status of the animal, as the demand for growth in the young animal is undoubtedly great. In the mature animal this demand to grow does not exist and the energy intake and expenditure are in a state of approximate equilibrium.

Evidence of a preliminary nature obtained in this work indicated a marked variation in heat production at different times of the day. Since the rat is a nocturnal animal, most of its activity would be expected at night or during the time when the laboratory was dark. It is therefore of interest to know something about this fluctuation in heat production

[1] Authorized for publicaton on September 18, 1948 as paper No. 1472 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

throughout the 24-hour cycle with activity unrestricted, as well as during the shorter periods which are used in the measurement of heat increments in which activity is limited. It seemed possible that the heat increment, measured under definite imposed conditions and for a part of the day only, might not represent the total daily dynamic effect actually produced under conditions of normal activity. Although considerable thought has been given this problem in the past, it appeared advisable to attack it again in connection with the present study of fat metabolism.

The existence of a diurnal metabolic rhythm in animals has been claimed by many investigators, with fluctuations in metabolism which are dependent mainly upon light cycles. Irritability, activity, muscular tonus, feeding times, et cetera, are factors which are influenced by light intensity, and these in turn alter the metabolic rate. The information on this subject appearing in the literature has been summarized by Brody ('45).

For the rat, Herring and Brody ('38) reported a diurnal metabolic rhythm with maximum variations of 25–30%. This evidence further indicates the desirability of investigating fluctuations in metabolism in relation to the problem under consideration.

EXPERIMENT NO. 1

Relation of fat to total heat production in the rat during 24-hour cycle with activity unrestricted

The specific purpose of the present experiment was to determine the changes in metabolic activity in the rat during a 24-hour cycle when diets containing either a low (2%) or high (30%) fat content were fed. The diets were compounded and fed so as to supply not only equal quantities of protein and energy but also essentially the same amounts of minerals and vitamins. The composition of the diets was practically the same as in previous studies on fat metabolism in this laboratory, and is given in tables 1 and 2 of an earlier paper by Forbes et al. ('46c). There was a slight difference in the

amounts of the protein and carbohydrate mixtures used, depending on the chemical composition of the ingredients in the mixtures. It is to be noted that the rations contained high levels of vitamins so that there would be no possibility of a suboptimum intake of any of these essential nutrients.

The subjects were 24 mature male rats, 16 to 17 months of age, with 12 rats in each of two groups. One group was fed the low fat diet, the other the high fat diet. The rats were selected from the same group of animals used in the previous work on this subject but were not necessarily the identical animals (Black, French and Swift, '49).

In an effort to have the same dynamic effect of the feed manifest on the curve of heat production during the day as at night, the daily allotment of feed was divided into two equal portions and fed exactly 12 hours apart, at 8 A.M. and 8 P.M.

The animals were thoroughly established on the experimental routine in the usual preliminary feeding period prior to a 10-day period for collection of feces and urine. The procedure followed for this collection was the same as that used normally in this laboratory and described in detail by Swift, Kahlenberg, Voris and Forbes ('34), except that a solution of cupric sulfate and sodium fluoride was used as a preservative in the crystallizing dish in which the urine was collected. The dish was washed out only at the end of the collection period.

The respiration measurements were conducted for two days following the collection period, this being done continuously for the 48-hour period in respiration chambers of sufficient size to accommodate the laboratory cage (Forbes, Swift and Black, '38). In order to determine the changes in metabolism throughout this time, ventilation of the chambers was stopped long enough every three hours to weigh the absorption tubes. This required only about three to 4 minutes on the average and was of such short duration that it did not permit any appreciable increase in CO_2 concentration in the respiration chamber. Any accumulation of CO_2 was thoroughly removed during the ensuing three-hour period. As the tubes from all

6 chambers could not be weighed simultaneously, it was necessary to stagger the time of weighings. This was done at 10-minute intervals, starting on the hour for the first cage. The actual times throughout the day when the weighings were made are the points indicated in figure 1, beginning at 1 P.M. and continuing every three hours thereafter.

Heat production was computed by the carbon-nitrogen balance procedure for each three-hour period, as well as the total for each of the two complete days. The method is described in detail by Forbes, Swift and Black ('38). The information on excretion of urinary and fecal carbon and nitrogen was computed from data comprising the average level of excretion for the 10-day collection period prior to the respiration measurements. For the three-hour periods three twenty-fourths of the average daily excretion was used, as it would have been impossible to make separate collections for such short periods.

Normal voluntary activity was permitted during the respiration measurements, the same cages being used during this metabolism test as during the rest of the experiment.

DISCUSSION OF RESULTS (EXPERIMENT NO. 1)

Table 1 presents the average daily intake and excretion of nitrogen. The values are in very close agreement with those reported previously using these rations (Black, French and Swift, '49), and again show that the level of fat did not influence the excretion or utilization of nitrogen under the circumstances imposed. The evidence indicates that the original objective, to feed at the maintenance level, was attained.

The difference of 17 mg in the daily intakes of nitrogen is due to unavoidable slight errors involved in analyzing and compositing the ration components while seeking to make the rations contain equal amounts of nitrogen and energy per unit of feed.

The data on daily intake and partition of energy, given in table 2, depict results which are quite similar to those obtained in previous work using this procedure (Black, French and

Swift, '49). Although the main objective was to show the hourly fluctuations in heat production, it was considered advisable to present the daily values as well since these figures relate the different experiments more closely. The daily heat production of the two groups of animals varies somewhat more than in any previous experiments but the variation is in the same direction as formerly, with the rats on the low fat

TABLE 1

Partition of average daily nitrogen intake per rat

FAT IN DIET	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION
%	*mg*	*mg*	*% of intake*	*mg*	*% of intake*	*% of intake*
2	512	471	92.0	462	90.2	1.8
30	529	495	93.6	485	91.7	1.9

TABLE 2

Intake and partition of daily food energy

FAT IN DIET	INTAKE	FECES	URINE	METABOLIZABLE ENERGY	BODY GAIN	HEAT PRODUCTION	
						Amount	Coefficient of variation
%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	%
2	57.43	2.50	4.04	50.89	5.21	45.68	4.93
30	57.42	2.15	4.25	51.02	7.35	43.67	2.43

diets producing more heat. The odds that this difference is not due to chance alone are 205 to 1, whereas the greatest significance obtained in any of our previous work using similar diets and techniques was characterized by odds of 38 to 1 (Black, French and Swift, '49). The tendency of a high fat diet to produce less heat than an equicaloric diet of low fat content is not as pronounced when the experimental conditions involve mature rats with unrestricted activity as it is when activity is restricted or young animals are used.

The curve of heat production extending continuously for a period of 48 hours and divided into three-hour intervals is presented in figure 1. It is apparent that the characteristic curves for the two diets showing the effects of the time of day and of the ingestion of food are definitely different in certain respects. On the low fat diet there was an increase in heat production after food was given either in the morning or evening, but the rise was much less during the day than at night. The increase during the morning seems to be largely dynamic effect, since it did not start until food was given,

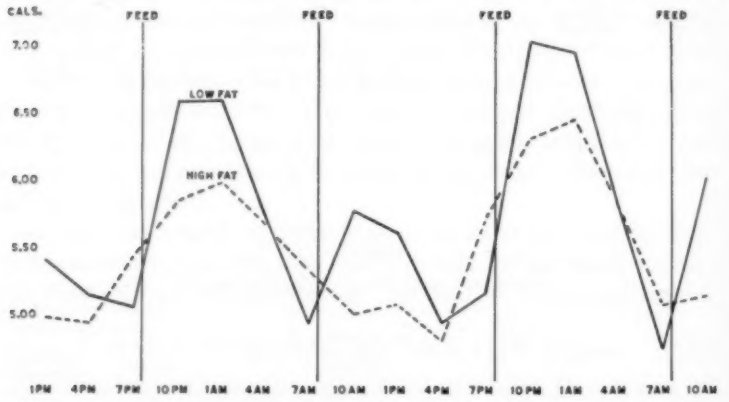

Fig. 1 Curves of heat production of rats on diets of high or low fat content.

while in the evening the increase started slowly between 4 P.M. and 7 P.M. This is obviously due to the activity of the rats, a fact noticeable to the observer during the hour prior to feeding. The peak of the dynamic effect and whatever extra activity there was during and immediately after eating the low fat diet was always reached within two hours after consuming the food, with a decrease in heat starting thereafter. This decrease was gradual at night, and even at 4 A.M., metabolic activity was greater than at any other time during the day.

The difference between the amount of activity during the day and night is clearly demonstrated by the animals on either

diet. There appears to be little activity by the rats on the high fat diet during the day, but after 4 P.M. an increase is evident which continues throughout the night.

The ingestion of the high fat diet had little demonstrable effect on the metabolic activity. This is in harmony with the results of the study of the dynamic effects of these diets (Forbes et al., '46b, '46d), in which it was found that the high fat diet had a much lower dynamic effect than did the low fat diet. At first glance the statement above does not appear to be entirely correct, since the heat production of the high fat rats rose to a great extent during the night. Part of the rise might be attributed to the heat increment of the evening feed, but this is contraindicated by the feeble rise in heat production following the morning feeding.

It is of particular interest and concern that the heat production of the rats on the high fat diet was greater at two specific times during the day than in the rats on the low fat diet, these times being between 4 and 7 P.M. and 4 and 7 A.M. Also the magnitude of the difference in metabolism between the two groups was greatest from 10 A.M. to 1 P.M., or during the time of day which had previously served as a basis for the study of heat increments. In other words, the conditions arbitrarily chosen for these experiments were those most likely to give a distinctly higher heat increment on a low fat diet. This problem has been investigated and is to be reported on in the second part of this paper.

Differences in gains of energy are accompanied by approximately equal and opposite differences in heat production (table 2). Results of previous work have also invariably involved a greater body gain of energy with accompanying lesser heat production on high vs. low fat diets. It is of interest to note that digestibility, metabolizability, dynamic effect or activity were not the factors directly related to gain of energy; only heat production has been so associated.

The metabolic rhythm noted in this study agrees to a considerable extent with the work of Herring and Brody ('38), in which they found fluctuations in the metabolism of the

rat varying from 25–30%. They measured metabolism by the Haldane procedure and doubtless the activity was restricted to a greater extent than in the work reported here, in which a rise of 40% in heat production was noted in the animals on the low fat diet. The influence of the type of diet on the daily metabolic fluctuation is apparent from the present study.

This work again points to the importance of considering the use of relatively long periods in the study of energy metabolism, as short periods may give no hint of factors prevailing at some other time of the day or at some point of a cycle other than the one in which the study is made.

EXPERIMENT 2

Relation of fat to heat production in the rat with activity restricted

In view of the results reported in the first part of this paper, showing the fluctuations in heat production at different times of the day in rats that were fed diets of varying fat content and permitted normal freedom of movement in the cage, it appeared of equal importance to learn what variation there might be in the metabolism when normal voluntary activity was restricted, for it is under this condition that the heat increment or dynamic effect has been determined.

This laboratory has been concerned with the problem for many years and has given much thought to it in planning experiments in which the heat measured was not to include that due to activity. The present experiment is by no means the first time the problem has been considered.

Although most investigators have for convenience used the daylight hours to measure the metabolic activity of rats, it has not been proved that this procedure is necessarily the most reliable. The present experiment was designed to produce further information on this subject, especially as the results might be influenced by the fat content of the diet.

The general routine of the experiment was the same as that outlined in an earlier paper (Forbes et al., '46d) with one ex-

ception, this being that the time of respiration measurements was shifted to begin about 1 P.M. and extend to 7:30 P.M. instead of starting at 8 A.M. and continuing to 3:30 P.M. This was done so as to include in the period of observation one of the times of day during which the heat production of the high fat rats was higher than the low fat rats under conditions of uncontrolled activity (fig. 1). This choice of time would be the least favorable to give a high heat increment on a low fat diet, a result previously reported.

Heat productions were determined by the Haldane respiratory quotient procedure as in previous similar experiments. Activity was limited by the type of respiration chamber as well as by a bright light placed over the chamber. To make possible the elimination of any intervals when activity was unavoidably high, the CO_2 production was determined hourly, and those hourly weights of CO_2 which differed from the average for the final 6 hours by more than $\pm$ 6% were excluded. The heat increment was determined as the difference in heat production of the same animals on maintenance and supermaintenance quantities of the same diets.

The subjects were two groups of 12 essentially mature albino rats about 6 months of age.

The diets were composed as previously reported (Forbes et al., '46c), one containing 2% fat, the other 30% fat. The rats on the 2% fat diet received 11 and 16 gm each of food at the two planes of nutrition, whereas the rats on the 30% fat diet received smaller quantities but isocaloric equivalents for the two planes of nutrition.

The schedule of experimentation was so arranged for each rat that a two-day maintenance respiration period began immediately after an 8-day maintenance excreta collection period.

Then followed a 5½ day preparatory feeding on the supermaintenance plane, a two-day period for respiration measurement on the supermaintenance feeding, and an 8-day excreta collection period on the same intake. This order of experimentation was followed so as to have the respiration periods

at the two planes of nutrition as close together as possible. In this way the weight and age of the animals were essentially the same at both planes of nutrition and correction for changes in live weight was unnecessary.

DISCUSSION OF RESULTS (EXPERIMENT NO. 2)

The digestibility of nitrogen (table 3) was essentially the same at both planes of nutrition, though slightly higher on the high fat diet at both levels of intake. No significant difference was found in retention of nitrogen, an observation consistent with results from previous experiments using these diets.

TABLE 3

Utilization of daily nitrogen

FAT IN DIET	PLANE OF NUTRITION	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION	
%		*mg*	*mg*	*% of intake*	*mg*	*% of intake*	*mg*	*% of intake*
2	Maintenance	392	364	92.9	387	98.7	—23	—5.9
30	Maintenance	394	371	94.2	395	100.3	—24	—6.1
2	Supermaintenance	570	530	93.0	485	85.1	45	7.9
30	Supermaintenance	573	538	93.9	490	85.5	49	8.6

The data on intake and partition of daily food energy (table 4), show little difference between the two diets in the energy of the urine or in metabolizable energy and only a slightly lesser amount of fecal energy on the higher level of fat intake. The percentage of metabolizable energy appearing as heat is less at both planes of nutrition on the high fat diet, indicating that the fat increased efficiency of utilization of the metabolizable energy in this work as in earlier experiments. The odds that the metabolizable energy of the 30% fat diet was more efficiently utilized than that of the 2% fat diet are 9600 to 1 at the supermaintenance plane of nutrition and 30 to 1 at the maintenance plane.

The heat productions as well as the heat increments (table 5) diminished markedly at the higher level of fat intake. The heat increments reported in an earlier paper were 6.16 and 3.29 Cal. for the 2 and 30% fat diets, respectively (Forbes et al., '46d). This represents a decrease of 47% from the low to the high fat. The same values in table 5 show a difference of

TABLE 4

Partition of daily food energy

FAT IN DIET	PLANE OF NUTRITION	INTAKE	FECES	URINE	METABOLIZABLE ENERGY		HEAT PRODUCTION		METABOLIZABLE ENERGY AS HEAT
					Amount	Coeff. var.	Amount	Coeff. var.	
%		*Cal.*	*Cal.*	*Cal.*	*Cal.*	%	*Cal.*	%	%
2	Maintenance	45.31	1.90	3.15	40.25	0.4	28.62	7.5	71
30	Maintenance	45.20	1.68	3.22	40.30	0.3	26.82	6.4	67
2	Supermaintenance	65.90	2.57	4.51	58.83	0.2	34.20	5.8	58
30	Supermaintenance	65.74	2.48	4.57	58.69	0.4	31.25	4.8	53

TABLE 5

Quantities, increments, and sources of average daily heat production

FAT IN DIET	PLANE OF NUTRITION	TOTAL HEAT AND HEAT INCREMENT	NON-PROTEIN R. Q.	SOURCE OF HEAT PRODUCTION			
				Protein	Carbohydrate	Fat	Fat synthesis
%		*Cal.*		*Cal.*	*Cal.*	*Cal.*	*Cal.*
2	Supermaintenance	34.20	1.17	12.85	20.58	0.00	0.77
2	Maintenance	28.62	1.08	10.26	18.06	0.00	0.30
2	Heat increment	5.58		2.59	2.52		0.47
30	Supermaintenance	31.25	0.89	12.98	11.73	6.54	0.00
30	Maintenance	26.82	0.85	10.47	8.29	8.06	0.00
30	Heat increment	4.43		2.51	3.44		0.00

21%. Some of the variation between the two experiments may have been due to the time after the last feeding when the heat increment was measured. In the first experiment the respiration measurement was started shortly after feeding, while in the present experiment it was not begun until 5 hours after feeding.

The main reason for the difference in heat increments between the two experiments is attributed, however, to the fluctuations in the curve of total heat production (fig. 1). The control of activity in the Haldane procedure is not perfect and it is conceivable that there might be sufficient movement in the animals, late in the afternoon, approximately to equalize the heat productions on the two diets and thus produce the same heat increments. It is gratifying that this did not occur, and even though the magnitude of the difference between heat increments was not as large as previously reported, the fundamental findings were satisfactorily verified. A knowledge of the fluctuations in metabolism during the 24-hour day is most useful in planning experimental work.

SUMMARY

Fluctuations in the curves of heat production for the 24-hour day for albino rats receiving equicaloric diets containing either 2 or 30% fat have been studied, using 12 rats on each diet. The heat production was measured by the carbon-nitrogen balance procedure at intervals of three hours each.

Characteristic differences in the curves of heat production for the two diets were observed. The influence of activity at different times of the day is clearly evident, as is the difference in dynamic effect of the diets.

Although fluctuations in metabolism were great among three-hour periods, the total heat for the day compared favorably with earlier results.

A further study was also made of the dynamic effect of the diets to determine whether activity had been a serious factor in these measurements. By choosing a time of day in which to make these determinations least likely to support

previous contentions as judged by the curve of total heat production, it was found that the high fat diet resulted in a much lower heat increment than the low fat diet, again confirming earlier findings.

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